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FURTHER STUDIES OF SOROSPORELLA UVELLA, A FUNGOUS PARASITE OF NOCTUID LARVÆ

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INTRODUCTION

Because of their peculiar mode of life and varied characters, the Fungi Entomogeni, or fungous parasites of insects, possess an unusual interest and have formed the subject of a number of classic papers in mycological literature. Not only are they of interest from a scientific point of view, but they possess an economic importance which is probably much greater than is generally supposed.

While the majority of mycologists are likely to overlook these fungi through a lack of familiarity with insects and their habits, it is equally true that an ignorance of mycology has usually led entomologists to give them at best but scant attention, even when their presence is evident. That they are often overlooked is due further to the fact that even in destructive forms their development usually takes place within the living insect. The externally visible growth which occurs, if at all, after death is usually more or less concealed because of the position of the host at this period; and in many instances it may be so evanescent that after a relatively short interval the cause of death can not be determined.

The fungi of this nature which are of economic importance belong for the most part to one of two categories, the Entomophthorales and Ascomycetales, while associated with the latter may be distinguished a third group of entomogenous "Fungi Imperfecti," which although assumed for the most part to be imperfect stages of ascomycetous forms, have not been definitely connected as yet with any perfect or acigerous condition.

In all the groups the economic value of the parasites concerned is due to the fact that their cycle of development may be a very short one and that in all cases an enormous provision is made for reproduction, a provision vastly greater than that of any of the many types of parasitic insects, an increasing number of which are now being used in an effort to control noxious forms.

[1] The writer wishes to express his appreciation to Dr. Roland Thaxter, of Harvard University, for helpful criticism of the work while it was in progress, and to Mr. W. H. White, of the Bureau of Entomology, United States Department of Agriculture, who first found the parasite.

The artificial propagation and dissemination of such fungous diseases in cases where they may be readily controlled is thus, evidently, a matter of no slight importance; and the necessity for a complete and exact knowledge of their development and life cycle in all cases is obvious.

Among the entomogenous forms, of which our knowledge is incomplete, *Sorosporella uvella* is of especial interest, not only on account of its peculiar characters, which appear to be quite unique, but because of its probable economic importance. It was first observed in Russia by Krassilstschik (*11*)[1] in 1886, and at intervals subsequently received a certain amount of attention from Russian investigators; but so far as the writer is aware it has never been recorded from other countries, despite the fact that it probably is widely distributed and destructive.

That it has not been more generally observed is without doubt due to the fact that fruiting bodies are not, as a rule, produced on the outside of the insects which are parasitized and that the insects disintegrate soon after death, leaving little that can be recognized.

It is evident from an examination of the scanty literature relating to the morphology and development of *Sorosporella uvella* that the information contained therein is very incomplete and often inaccurate, and that little or nothing is known of many phases of its development, pathogenicity, prevalence, range of hosts, etc. The object of the present paper, therefore, is to give as complete an account as possible of the stages in the life history of the fungus which have previously been neglected or inaccurately described, as well as to consider certain hitherto unrecorded phases, some of which are of considerable importance from the taxonomic, pathogenic, and even economic point of view.

It will be shown further that one type of blood corpuscles of cutworms acts as phagocytes, in that they engulf and apparently attempt to destroy the vegetative bodies of the fungus. So far as the writer is aware, the phenomenon of phagocytosis in insects attacked by fungous diseases has been observed by no one, with the possible exception of Metchnikoff (*14*), and is of especial interest, therefore, in the present connection.

The observations herewith recorded are based on studies of artificial cultures of the organism, on inoculation experiments with living hosts, and on a histological study of infected insects prior and subsequent to death.

HISTORICAL SUMMARY AND TAXONOMY

So far as the writer has been able to determine, the first description of the form under consideration is that of Krassilstschik (*11*) in Russia, who in 1886 discovered the organism within the larval body of the coleopterous sugar-beet curculio, *Cleonus punctiventris*. He referred the fungus to Tarichium, which, although it has been considered by some writers even in recent years to be a valid genus of the Entomophthorales, is in reality

[1] Reference is made by number (italic) to "Literature cited," p. 438-439."

merely a name applied to species of Entomophthora in which only resting spores are as yet known.

Although Krassilstschik's description of *Tarichium uvella* is meager and unaccompanied by figures, the measurements and characteristics of the singular resting spores which are peculiar to this parasite are clearly recorded. Those bodies are described as spherical, papillate, and from 8 to 10 microns in diameter. They are said to cohere in grapelike clusters, and to form a brick-red mass within the bodies of the dead insects. Upon germination in a nutrient solution they gave rise to septate germ tubes which bore single, terminal, cylindrical, colorless spores measuring 9 by 3 microns.

It is evident that the method of germination of the resting spores described above does not coincide with analogous processes in the Entomophthorales. Furthermore the brevity of the description served to confuse rather than aid subsequent systematists who were inclined to follow Krassilstschik in referring the organism to this family.

Two years later Sorokin (*17*, *18*), another Russian writer, found a peculiar organism in the larva of a lepidopterous insect, *Agrotis segetum*, and, apparently unaware of Krassilstschik's work, described and figured what is without doubt the same form, under the name *Sorosporella agrotidis*. Sorokin's figures are sufficiently complete and his description of the resting spores sufficiently comprehensive to render an identification possible and to indicate clearly that the form with which he was concerned was identical with that previously described by Krassilstschik.

The characteristic coherence of the resting spores in masses or aggregations was recognized by both of these authors in naming the organism, Krassilstschik expressing the condition by using the specific name *uvella*, while Sorokin selected "Sorosporella" to designate his new genus.

In 1889 Giard (*8*) called attention to the similarity of *Tarichium uvella* Krassilstschik and *Sorosporella agrotidis* Sorokin, pointing out that the characters of the two forms, such as the reddish-colored, powdery spore mass, the peculiar coherence of the resting spores, and their measurements (according to Krassilstschik, 8 to 10 microns; according to Sorokin, 4 to 7 microns) corresponded so closely that he believed them to be identical. Giard, however, in agreement with the majority of mycologists at the present time, held that the generic name Tarichium, of which the type is *T. megaspermum* Cohn., should be confined, if employed at all, to those species of Empusa and Entomophthora in which resting spores only are known. As the resting spores of the form under consideration are entirely unlike those of any known Empusa or Entomophthora, he proposed that the generic name Sorosporella be employed together with the specific name *uvella* of Krassilstschik. Giard, however, while of the opinion that the fungus should not be called Tarichium, adhered to the belief that it should be placed in the Entomophthorales.

Since the name *Sorosporella uvella* (Krass.) Gd. conforms to the present nomenclatorial rules, it will be used in this paper to designate the organism; but, as will be subsequently shown, its application to the Entomophthorales by Giard and others was undoubtedly erroneous.

Since the publication of the two papers above mentioned, descriptions have appeared of three other fungi which are perhaps identical with *Sorosporella uvella*.

Bresadola (*3*) described an entomogenous parasite under the name of *Massospora staritzii*. No illustrations were published, and the host was given as the larva of an unknown insect. The "conidial" mass was said to have been endogenous, pale flesh-colored, and the measurements of the globose or subglobose "conidia," which were provided with slightly roughened epispores, were given as 9 to 11 by 7 to 9 microns. Bresadola was unable to determine the method of formation of the bodies which he termed "conidia" because the specimen was too old.

In the following year Giard (*9*) published a note on *Massospora staritzii*, in which he expressed the opinion that this species bore considerable resemblance to *Sorosporella uvella* and suggested that the latter name be considered a synonym.

Although Bresadola's description is quite incomplete and does not mention a grouping of the resting spores, such as is characteristic of Sorosporella, it seems quite likely that *Massospora staritzii* is indeed a Sorosporella, but on account of the color of the fungus mass, which differs considerably from that of *S. uvella*, as well as the presence of a roughened epispore, the writer is inclined to consider it a different species. At this point it may be stated that the writer has in culture at the present time a species of Sorosporella, isolated from the adult of the coleopterous *Ligyrus gibbosus*, in which the fungus mass is white, and although not carefully studied as yet, its characters seem to conform more readily with those of *Massospora staritzii*. The name used by Bresadola is therefore mentioned in the present connection as a possible synonym, in order that its probable relation to the genus Sorosporella may not be overlooked.

As a second species, *Acremonium cleoni* was described by Wize (*24*) from the larva of the coleopterous insect *Cleonus punctiventris*. While Wize seems to have been familiar in a general way with *Sorosporella uvella*, which is mentioned in the introduction to his paper, he appears to have been quite unfamiliar with its development, especially the processes connected with resting-spore germination. According to Wize, *Acremonium cleoni* develops in the larvæ and pupæ of Cleonus, forming spherical, cohering cells 8 microns in diameter. From these are produced, on the outside of the insect's body, branched, septate hyphæ, some branchlets of which are bottle-shaped and bear single, terminal, ellipsoid, hyaline conidia measuring 6 by 3 microns. In artificial culture the fungus reproduces in a yeastlike fashion, first forming yellowish cells from which grow structures similar to those found on the outside of the insect's body

and bearing similar conidia. In the following description of *Sorosporella uvella* it will be seen that the method of development, including the internal formation, coherence, and size of the resting spores, the yeastlike reproduction in culture, the character of the conidiophores, the shape and size of the conidia, etc., so readily conform with analogous characters of *Acremonium cleoni*, which, furthermore, parasitizes the same insect, that there can be no doubt as to the identity of the two species.

A third species, *Fusarium acremoniopsis* Vincens (*22*), should be considered in this connection; and while the measurements of the bodies which Vincens terms its chlamydospores (3.5 by 4 microns) as well as the measurements of its conidia (4 by 7 by 2 by 3 microns) do not coincide with similar structures of *Sorosporella uvella*, there are nevertheless many points of resemblance between the two fungi which cannot be disregarded and which show, in the writer's opinion, that they, though perhaps not identical, are at least very closely related.

Vincens described his fungus as a parasite of the "ver gris" (cutworm?) from Brazil. The insect when dead was dark brown in color, but no external fructification was evident. Internally, however, Vincens found mycelia bearing short, undifferentiated conidiophores at the tips of which cylindrical, one-celled conidia were produced singly. His illustration of such hyphæ is very similar to certain structures found by the writer when *Sorosporella uvella* was grown upon artificial nutrients (Pl. 52, O). It must be noted, however, that no such structures were ever observed in cutworms infected by the same fungus.

Vincens placed fragments of the caterpillar in a moist chamber; and from these fragments conidiophores developed, the branches of which had a tendency to group themselves verticillately, a tendency, it will be shown, that is noticeable also in the conidiophores of *Sorosporella uvella*. The cylindrical spores borne on these conidiophores were largely unicellular, but a certain number were curved and provided with one or more septæ.

At a later date Vincens discovered, in dried-out fragments of the caterpillar, large numbers of brown, globular spores, grouped in spherical or oval masses, the illustrations of which resemble very closely the resting-spore aggregations of *Sorosporella uvella*, which, as the writer has indicated, are distinctive bodies and serve at once to differentiate Sorosporella from all other entomogenous fungi.

There is, therefore, considerable resemblance between *Fusarium acremoniopsis* and *Sorosporella uvella;* and in spite of the fact that the measurements of their respective reproductive bodies are dissimilar, as well as the fact that the conidia of *F. acremoniopsis* are said to be sometimes septate, the writer believes that Vincens' fungus, though perhaps not *S. uvella*, is at least some closely related species.

A few other papers, chiefly in the form of taxonomic notes, such as those of Thaxter (*21*), Danysz and Wize (*5*), and Lakon (*12*), have

appeared, in which attempts are made to define the relationship of Sorosporella with other entomogenous fungi, but on account of the scanty information afforded by the literature on the subject and the lack of original work such attempts have not met with much success.

It has been generally believed up to the present time, however, that the fungus under consideration should not be called Tarichium in the sense that the name is employed in *T. megaspermum*, but that nevertheless it should be placed in the Entomophthorales because of the resemblance of the resting spores to those of the entomophthoraceous genus Massospora.

In addition to such purely taxonomic papers, one or two have appeared dealing mainly with the artificial culture of the organism and its value as a means of combating insect pests.

Skrzhinskiĭ (*16*) apparently observed the yeastlike vegetative development of the fungus within the insect; but the significance of such a method of development was evidently not appreciated, for it was but briefly mentioned.

Danysz and Wize (*6*) report success in cultivating the organism on artificial media but deduce several erroneous conclusions regarding its life history, stating that the resting spores multiply by division within the interior of their walls, which afterward burst and release the newly formed spores. The latter continue to grow, subdividing into two or four if conditions are favorable; but if the nutrient in which they are growing becomes dried out, thick walls are produced which render them identical in appearance with the mother cells. If the cultures are kept for a sufficient time, mycelial filaments are produced from the encysted cells. Nothing is said, however, of the conidia which probably arise on the filaments. In this connection it should be stated that these authors were unable to inoculate insects either with the resting spores or with the mycelial filaments, yet they admit the infectiousness of the fungus in stating that in certain regions of Russia it is a more effective enemy of Cleonus than is *Metarrhizium anisopliae* (Oospora).

So far as the writer is aware the foregoing abstracts include all the more important references to *Sorosporella uvella* which have appeared up to the present time, and from the information therein it is obvious that the life history of the organism has been incompletely known, rendering it one of the most obscure forms of the entomogenous fungi.

Since all the descriptions of the fungus available at the present time are so brief as to be almost valueless for taxonomic purposes, the following is appended:

Sorosporella uvella (Krass.) Gd. (*5*) Syn.: *Tarichium uvella* Krassilstschik (*11*); *Sorosporella agrotidis* Sorokin (*17*); *Acremonium cleoni* Wize (*24*); ? *Massospora staritzii* Bresadola (*3*); ? *Fusarium acremoniopsis* Vincens (*22*).

Entomogenous. Resting spores formed endogenously, spherical or subspherical, 7 to 10 microns in diameter, occasionally papillate, with somewhat thick, irregular

walls; cohering in groups and arising from yeastlike, hyaline, elliptical budding cells. In mass, brick-red in color ("Brick Red" Ridgway) and powdery. Conidia thin-walled, hyaline, 4 to 6 by 9 to 11 microns in diameter, abjointed successively from bottle-shaped or almost subulate branchlets of simple or branched conidiiferous septate hyphæ and adhering after abjunction.

Hosts: *Cleonus punctiventris* Germ. (Coleoptera), Russia; *Agrotis segetum* Esp. (Lepidoptera), Russia; *Euxoa tessellata* Harr.; *Noctua c-nigrum* L.; *Agrotis ypsilon* Rott.; *Feltia subgothica* Haw.; *F. jaculifera* Guen.; and various other cutworms and noctuids in the eastern United States and Canada.

LIFE HISTORY

An opportunity was first afforded the writer in June, 1916, to study this fungus on infected cutworms received from College Park, Md. The insects showed no external signs of fungus attack even though dead, but when they were broken open a brick-red, powdery mass escaped from the larval shell (Pl. 51, A) which when examined microscopically was found to consist of spherical or subspherical, somewhat reddish-colored, moderately thick-walled cells.

Attempts were made at once to cultivate the fungus on artificial nutrients, and the usual plate isolation method was employed with potato agar as a medium. The results of the first tests were somewhat discouraging, but finally a pure culture was obtained, on another nutrient, from which subcultures have been made continuously since 1916. The following discussion is therefore based upon a study of diseased insects collected in the field, as well as on a study of insects that were inoculated in the laboratory from cultures on artificial nutrient media.

The thick-walled spherical cells as they occur within the host may be solitary, but more often they cohere in characteristic masses or aggregations (Pl. 51, H). Many show wartlike protuberances (Pl. 51, E), and others show coherent fragments of the walls of cells to which they were previously united (Pl. 51, E).

As will be shown in another connection, they may retain the power of germination for a considerable period because of their rather thick walls; and since they are, therefore, functionally analogous to similar thick-walled cells of the Entomophthorales and many other fungi, they may be termed resting spores or chlamydospores.

If a water mount is made of these resting spores and pressure is applied to the cover glass, it becomes evident that the individual cells are very firmly coherent, since their association is broken up with considerable difficulty. If sufficient pressure is applied, however, the homogeneous character of the resting-spore masses becomes apparent. The masses are resolved into their constituent cells, which prove to be undifferentiated and uniform throughout.

It should be stated in this connection that specimens collected in the field are found almost invariably in the condition just described, because the resting spores, which mark the termination of the development of

the fungus, may remain dormant for considerable periods, and when germination takes place the larval body has usually disintegrated to such an extent that it is no longer recognizable. All accounts of the fungus up to the present time have, therefore, been concerned mainly with the resting spores, although as a matter of fact these bodies represent but one phase in the rather complicated development of the organism, while an equally important phase, the production of aerial conidia, has usually been overlooked or at least not associated with the chlamydosporic condition.

Since the resting spores are thick-walled and occur within the unbroken body wall of their host, which as a rule appears to die beneath the surface of the ground, and since they are freed only by the disintegration of the host, it is obvious that they are not adapted to propagate the disease with rapidity by carrying the fungus from insect to insect, but are rather designed to enable it to survive drought and other unfavorable conditions.

To determine the origin, nature, and function of the resting spores, a number of experiments have been performed during the past two years.

In order to induce germination, freshly collected dead larvæ were placed on the surface of moist sand in covered crystallizing dishes and kept at room temperature in the laboratory. Three days later an external fungous growth on the unbroken integument of the host was distinctly visible, and after an interval of one week the latter was found upon examination to be composed of large numbers of conidiiferous hyphæ of the type illustrated in Plate 51, L. The successive stages in the germination of the resting spores and the development of the conidiophores are shown in Plate 51, F, L, N, O. Under the stimulus of moisture and suitable temperature the protoplasm of the resting spores swells, producing budlike protuberances, the walls of which are in part, at least, made up of the walls of the resting spores. The outgrowth soon assumes the shape of a germ tube, branches freely, and becomes septate. The fully developed conidiophores are supplied with bottle-shaped branchlets which show a tendency to group themselves verticillately around the main hypha. It will be further observed that conidia are borne at the tips of the bottle-shaped branchlets or sterigmata which are quite unlike the resting spores from which they arose. They measure 4 to 6 by 9 to 11 microns, are elliptical in form, thin-walled, vacuolate at each pole and are abjointed successively in the manner illustrated on Plate 51, P, cohering after abjunction.

The development and structure of the conidiophores as well as the formation of the conidia are typical of the verticillate Hyphomycetes, of which group Sorosporella should be considered a member until its perfect or acigerous condition is discovered.

The occurrence of the chlamydospores in coherent groups or aggregations recalls a similar condition that is found in the spore balls of

certain of the smuts, such as Urocystis and Tubercinia, a resemblance noted by Sorokin (*17*) in 1888. Furthermore, early stages in the germination of the resting spores in hanging drops of water (Pl. 51, C) are similar to analogous processes of promycelium and sporidium formation in some of the Ustilaginales; but although this resemblance is rather striking, it is purely superficial and of no phylogenetic significance.

The resting-spore aggregations are more naturally comparable to that class of propagative bodies known as bulbils, which, as Lyman (*13*), Hotson (*10*), and others have shown, occur in the life histories of certain Ascomycetes and Basidiomycetes. Their method of development, however, is quite different.

So far as can be determined from his imperfect description, something similar to the conidiophore production apparently was observed by Krassilstschik (*11*); and this phase of development is in itself sufficient to show that the organism can not possibly be included in the Entomophthorales, since the coenocytic hyphæ of the latter, together with the peculiar method by which their conidia are formed and discharged, are radically different and in no way comparable to corresponding characteristics of the form under consideration.

The germinations thus obtained from unbroken larvæ treated as described above were found to be confined to those spores only which lay near the surface, immediately beneath the integument. When, however, the infected larvæ were torn open and the red spore masses freely exposed to the air and light, a somewhat different result was obtained in several instances. Under these conditions the germination involved practically all the spores composing the mass, and a luxuriant growth developed quite unlike that obtained in the first instance. The differences observed were due to the fact that germ tubes from adjacent chlamydospores cohered in such a way that Isaria-like fascicles of conidiophores were produced (Pl. 51, G). Conidia were formed in the same manner and are apparently identical in every way with those described above. This type of growth does not invariably appear when infected larvæ are treated as described, but it is not unusual. The larvæ died but a few days before the tests were performed, so the resting spores were comparatively young and fresh.

The Isaria-like grouping of the conidiiferous hyphæ is somewhat similar to that which occurs in certain species of the genus Cordyceps, a condition that may be permanently conidiiferous, or represent a stage preliminary to the perfect or acigerous phase of the development. Such a condition, which was found to be parasitic upon leafhoppers (Perkinsiella) and other insects in Hawaii, and which is similar in many respects to *Isaria saussurei* that attacks wasps, has been described and figured by the writer (*19*) under the name "sterile Cordyceps." Furthermore, it has been suggested by Cooke (*4*) that *Isaria saussurei* is the imperfect

stage of *Cordyceps sphecocephala*, and the occurrence of an analogous structure in Sorosporella suggests that it also represents the imperfect stage of some species of Cordyceps or an allied type.

A second series of infections was made with army worms, *Cirphis unipuncta*, and as in the instance described above, freshly dead insects were employed. The artifically infected larvæ were, however, nearly all in the pupal stage at the time of death, the pupa case forming a thick, resistant, chitinous envelope about their dead bodies. Such pupæ were placed in moist chambers at periods varying from one week to two months after death; and although they were completely filled with resting spores which were quite normal in appearance, as was shown by subsequent examination, they produced no external conidiophores, nor did any of the resting spores germinate. When the pupa cases were torn open, however, permitting a free circulation of air about the fungus, germination was readily induced.

The results of the foregoing experiments show, therefore, that the resting spores or chlamydospores do not necessarily require a long period of rest before germination, a period that may be necessarily more or less protracted in certain other fungi, but that if suitable conditions obtain, germination may take place at once. The tests also show that when insects die in the pupal stage, germination does not take place at once unless the thick chitinous wall is broken.

In order to determine the longevity of the resting spores as well as to ascertain whether or not fresh insects could be infected with them, other tests were performed as outlined below.

Infected cutworms which died in June, 1916, were kept in the laboratory in ordinary pasteboard pill boxes until August 2, 1917, when a number of resting-spore masses were removed from them and placed in hanging drops in Van Tiegham cells. On August 6, they began to germinate; and subsequently conidiophores and an abundance of conidia were formed. This result demonstrates that the resting spores may be viable after they have been subjected to desiccatory conditions for 14 months.

In October, 1917, a number of infected army worm pupæ were buried out of doors about 2 inches beneath the surface of the soil. On March 8, 1918, they were exhumed and examined. So far as could be determined microscopically, no change had taken place; they appeared just the same as when buried the autumn before. One pupa was broken open and spore masses placed in Van Tiegham cells, while the pupa itself was placed in a moist chamber in the laboratory. On March 11 the resting spores in the Van Tiegham cells began to germinate, and conidiophores could be seen with the hand lens on the pupa in the moist chamber. The winter of 1917–18 was exceptionally severe, yet the chlamydospores were apparently unharmed. Their germination shows clearly that they are able to withstand cold as well as desiccation.

To determine whether or not fresh larvæ could be infected with newly formed ungerminated resting spores, 10 cutworms were allowed to crawl over and were rolled about in a finely powdered layer of chlamydospores in a Petri dish for 15 minutes on June 7, 1917, after which they were removed to sterile dishes in which there was a small amount of sterile moist sand. By June 25 six of the larvæ had pupated, afterward emerging. One died, but apparently not from the Sorosporella disease. On June 20 the fungus was discovered in one dead pupa, and on July 2 in another. One insect escaped during the feeding interval following inoculation. Since the larvæ were placed after inoculation in a sterile covered dish, the cover of which was removed only when fresh food was inserted, the possibility of infection from other sources seems rather remote. Although a low percentage (2 per cent) of the larvæ died from the disease, it should be noted that the deaths occurred on the twenty-third and twenty-fifth days after inoculation, whereas the normal incubation period is from 10 to 12 days, as will be shown later. As will be explained below, there occurs within the blood of infected cutworms a phagocytic reaction which may have inhibited the development of the disease, thus tending to protract the incubation period; but it seems more reasonable to believe in the present instance that infection was secured, not through the resting spores directly, but by conidia which they produced after germination; and it is obvious that the period of time necessary for the germination of the resting spores and for the production of conidia which may or may not have been in position to infect larvæ immediately after formation would readily conform with the results of the test.

Several important facts become apparent as a result of such tests, some of which appear to have a definite economic bearing. It has been shown that the chlamydospores are formed for the purpose of tiding the fungus over unfavorable conditions, since they are thick-walled and are able successfully to withstand desiccation as well as out-of-door winter temperatures. Furthermore, upon germination in hanging drops of water or in a moist chamber, they do not produce simple undifferentiated germ tubes as would be expected under these conditions, but complexed branching conidiophores, which in turn give rise to thin-walled conidia.

Although the primary function of the resting spores is that described above, it has been shown that such bodies do not necessarily require a long period of rest but may germinate soon after their formation if freely exposed to moist air. In this respect they are similar to the spores of the Ustilaginales and to the bulbils.

It is reasonable to conclude, therefore, that several generations of the fungus may occur during one season under field conditions in spite of the fact that resting spores exclusively are formed at the close of the vegetative development of the organism. This is all the more probable

because most species of cutworms habitually burrow in the soil, where they come in contact with the conidia, and because the generations of different species and even of the same species overlap and are susceptible to the disease as has been shown by inoculation experiments.

The results of the test with the army worm pupæ indicate, however, that when the resting spores are formed and retained in a thick, intact, resistant, chitinous wall such as the insect forms at this period, germination will not take place; and in fact development of conidiophores is much less luxuriant upon the untorn body wall of a larva, which, however, becomes very thin and parchment-like after a time, than on one which has been torn open. This difference is well shown on Plate 51, K, and Plate 55, A.

It is, therefore, apparent that although the germ tubes have the power of breaking out through very thin chitin, the process of germination is undoubtedly facilitated greatly when larvæ become disintegrated in the soil.

The conidia which are borne at the tips of the germ tubes are, as stated above, of a different nature from the resting spores. Their thinner walls and formation aerially in enormous numbers, as well as their general ephemeral appearance, indicate that, like analogous conidia of other Hyphomycetes, their function, quite unlike that of the resting spores, is to spread the organism rapidly and as completely as possible while favorable conditions obtain.

In a water drop, or in a drop of nutrient agar in Van Tiegham cells, germination of the conidia takes place as shown on Plate 51, J, by the production of threadlike, sinuous germ tubes. Experiments have shown (p. 433) that when conidia are placed in contact with healthy insects, either extêrnally or internally, infection may be readily induced; and it is probable that germ tubes similar to those mentioned above penetrate the thinner portions of the body wall from without, or from within through the intestine, producing at their tips bodies which give rise to yeastlike vegetative cells. The exact method of infection, however, has not been determined, in spite of the fact that a large number of insects have been killed, fixed, and sectioned in attempts to observe germ tubes actually penetrating the insect tissues.

In the foregoing discussion the morphology of the single-walled resting spores as well as their functions and germination has been considered. It has been shown that they produce conidiophores, which in turn produce the thin-walled conidia. The production of conidia evidently marks the termination of the reproductive stage, all phases of which occur after the death of the host. The remaining stages, except the formation of the chlamydospores, are vegetative in nature and are found within the living insects after infection.

Cutworms parasitized by *Sorosporella uvella*, like insects attacked by other fungous parasites, exhibit no symptoms of disease during the

first few days of the incubation period. Three or four days prior to death, however, they become sluggish and eat sparingly; and one or two days before death a change in outward appearance may be observed. The chitinous body wall of healthy larvæ of *Feltia annexa*, one of the hosts employed, is dorsally opaque and flecked with irregular dull brown patches. Ventrally it is somewhat translucent, in fact so much so that some of its internal organs are visible. When afflicted with this disease the larvæ typically turn a creamy white color a day or two before death. If the flaccid body is pricked with a needle, a greenish or whitish liquid appears in which, if it is examined microscopically, yeastlike cells can be readily detected. These cells may be formed in enormous numbers and when abundant in the insect cause the blood to appear white, while if there are few yeast cells present the normal green color of the blood prevails.

Occasionally red-colored patches appear a few hours before death, either posteriorly, anteriorly, or, more commonly, near the middle of the body. They are more noticeable on the ventral surface because of the lack of pigment in this region. Dissections show that mature resting spores are present in such areas; and their early occurrence and maturity in such spots possibly indicate the seat of infection, although, as noted below, the detached yeastlike cells which later develop into resting spores are distributed throughout the body cavity by the blood of the insect in such a way that a local early maturity of the chlamydospores would seem impossible.

In one or two instances the whole middle portion of the body of an infected insect became reddish and noticeably shrunken, the anterior and posterior ends retaining the normal greenish, turgid appearance. The prolegs of such diseased portions showed no reaction when pinched with a pair of forceps, but the true legs at the anterior part of the body reacted when similarly treated.

With the exception of such symptoms, which, as has been stated, occur only in the later stages of the disease, there are no others prior to death, so far as the writer is aware, which indicate the presence of Sorosporella.

Death follows a day or two after the natural pigment of the insect disappears; and the creamy white color soon changes to pink, the latter usually appearing simultaneously over the whole body and becoming more and more intense until the final development of the organism, indicated by the brick-red color, is reached. The disease can thus be readily recognized at any time after death without the aid of a microscope.

In contrast to most known species of entomogenous fungi (except certain species of Entomophthora which form resting spores only), Sorosporella completes its entire development within the body of its host, producing no growth externally.

Shortly after death the body appears shrunken and wrinkled. It is somewhat flattened, and there is nearly always a longitudinal, ventral,

furrow-like depression present in its abdomen. The body, while not limp or flaccid, is soft and pliable; and if a portion of its skin is indented, it remains sunken with little or no reaction. The body is never hard and sclerotium-like as in insects infected with *Botrytis rileyi*, for example. If pricked with a needle no liquid emerges. If torn open entirely, the internal fungus mass is seen to be quite coherent and of a shiny creamy or pink color and gelatinous consistency. A convoluted, almost vermiform structure of the spore masses can easily be recognized with a hand lens.

Later in the development of the organism the host's body becomes more shrunken and the reddish color more intense, but there is no other change in outward appearance. The body wall is now quite brittle, the slightest shock serving to rupture it. The moist, gelatinous character of the spore masses has disappeared; and they become typically brick-red in color, dry, dustlike, and less coherent than before. Absolutely nothing remains of the internal organs of the host, and in fact the body appears as, *17* Sorokin (*18*) first suggested, very much like a minute sac filled with dust.

The fungus is found in this stage of development when collected in the field in summer, and according to Danysz and Wize (*6*) it may be seen in the same condition after having overwintered in the soil, a statement, it will be recalled, that is in accord with the writer's findings.

In the foregoing paragraphs the known symptoms of diseased insects, as well as the gross characters of the fungus during the incubation period and the post-mortem aspects of the disease, have been considered. To observe the microscopic characters of the vegetative stages of the fungus, however, many infected specimens have been examined when alive and when killed, fixed, and sectioned. During the first few days of the incubation period it is quite impossible to recognize the organism in any of the insect tissues. Upon examination of the blood of infected insects on the sixth or seventh day after inoculation, however, yeastlike cells will be observed floating free in the blood lymph mingled with the blood corpuscles. These yeastlike cells, as will be subsequently shown, form the early vegetative states in the development of the organism; and on account of their similarity to yeasts, the name blastocysts will hereafter be employed to designate them.

The cells when young are quite regularly elliptical in form, hyalin, measuring 8 microns by 5 microns, and in blood smears occur singly or coherent in pairs, rarely in threes; (Pl. 52, I; 53, B). A preparation made from the blood of a diseased insect on the sixth day after inoculation will show but few of these bodies, but on the seventh day they become more numerous, and on the eighth and ninth days, under normal conditions, they are strikingly conspicuous, vastly exceeding in numbers the blood corpuscles, which may themselves be abundant. They multiply within the blood plasm by a yeastlike germination, all stages of

which can be seen in one preparation. Sometimes at one end, sometimes at both ends of the blastocyst a budlike outgrowth or papilla appears which grows rapidly until it assumes the form and approximate size of its parent. For reasons given below it soon breaks away from the parent cell and proceeds to form new cells by a similar process. At times a mucronate outgrowth appears at the tip of the blastocyst instead of a papilla, so that a short neck is formed, at the distal end of which a daughter cell arises (Pl. 52, I).

Multiplication continues in this manner until enormous numbers of blastocysts occur within the blood plasm; and since they are free-floating, the circulation of the blood distributes them uniformly from place to place, so that they are found throughout the body cavity, within the heart (Pl. 55, B), and in all regions where it is possible for the blood to penetrate. The nature of the blastocysts and their somewhat loose attachment to one another is such that the circulation of the blood must evidently be considered the essential factor in spreading them to all parts of the body as well as in aiding vegetative reproduction.

The blood lymph becomes so loaded with blastocysts that not only is its progress eventually impeded but its function of supplying oxygen to the organs of the insect is apparently inhibited. As a result of what is perhaps lack of oxygen, the host acts as though stupefied; and, though alive, it responds to stimuli feebly, remaining in a comalike condition for several hours before death.

When the insect dies or when the blood circulates slowly before death, the blastocysts, though formed in the usual manner, no longer break away from one another; instead of isolated cells, colonies of coherent individuals are formed (Pl. 51, D), which ultimately develop into the resting-spore masses. At the same time that the colonies are formed, however, the blastocysts individually change from an elliptical to nearly spherical form. Since each blastocyst is able potentially to form new colonies, a large number of the latter are produced; but many do not grow to large size, evidently because the nutrient in the blood is exhausted. Other small colonies coalesce so that large spore masses are formed, and still others that are in close proximity to fat bodies or other sources of nourishment are able to grow as long as this nutrient lasts.

The writer has never seen cells of the parasite actually intruding into the insect organs, and in fact the budding process of growth is such that intrusion would not be expected. On the contrary there appears to be a substance secreted by the fungus which causes the organs to break down; and, as would be expected, those which are in contact with the fungus colonies disintegrate first.

The chitinous body wall of larvæ in which resting spores are fully mature is always very thin (Pl. 51, K), and even the tracheæ are broken down completely, fragments of the taenidia only remaining; whereas in insects in which blastocysts are beginning to develop the tracheæ are

intact, and the body wall, except after molting, is very thick. When resting spores are transferred from freshly opened larvæ to artificial media pure cultures are invariably obtained, indicating that no extraneous organisms are present in the body cavity; and the fact that the chitin has a tendency to disintegrate in the presence of Sorosporella indicates that this fungus secretes a solvent of some kind.

Before the chitin breaks down, however, all the softer tissues disappear, among the first of which are the fat bodies, the membranous walls of which disintegrate readily (Pl. 52, A).

A colony of fungus cells which is closely opposed to a fat cell will bud off more freely nearest the source of nourishment; hence, after the wall of the fat body has broken down at the surface, the colony will tend to enter and take the form of the organ, a portion of the wall of which persists for some time. The result of such development is that lamella-like or vermiform convoluted colonies are formed.

Although the fat bodies disappear first as a result of the growth of the fungus, the muscles, nerve fibers, malpighian tubes, and all the hypodermal tissues gradually succumb, until eventually only fragments of the tracheæ can be observed. Even the alimentary tract disappears, and its position is indicated merely by fragments of food.

As the colonies of the fungus continue to grow, the individual cells gradually become larger and secrete single, rather thick walls about themselves, so that ultimately aggregations of spherical, rather thick-walled, cohering cells are formed, which were described above as resting spores or chlamydospores. The formation of resting spores marks the end of the vegetative development of the fungus. These spores are in fact only modified blastocysts, though they are themselves reproductive.

The discovery of the yeastlike cells or blastocysts, the method of development and mode of life of which have been considered above, and the fact that they represent the entire vegetative stage of the organism under consideration, is of considerable scientific importance. Although found by the writer for the first time in connection with Sorosporella, similar bodies were observed by De Bary (*2*) associated with other entomogenous fungi, a fact quite unknown to the author until these investigations were completed.

De Bary found what he called "cylindrical conidia"—processes analogous to what have herein been called blastocysts—free-floating within the blood of various insects infected with *Botrytis bassiana*, *Isaria farinosa*, and other similar fungi. He observed that these bodies, though sometimes drawn out to two or three times their original length, usually remained elliptical in form and gave rise to secondary and tertiary conidia on short sterigmata. Such a method of multiplication was followed until the blood at the expense of which the organism developed was full of "cylindrical conidia," when, because of the large numbers of

these bodies present, it became milky white in color. He also noted that the circulation of the blood was an important factor in the distribution of the blastocysts, and that it gradually became absorbed, so that when an insect was pricked with a needle in later stages of the disease no liquid emerged. When the cylindrical conidia had completed their development, however, instead of rounding up to form chlamydospores, as do the similar bodies of Sorosporella, the bodies were observed by De Bary to lengthen out, become septate and branched, and otherwise assume the form of branching hyphæ.

It should be stated, however, that the "cylindrical conidia" were considered by this author to be homologous with those formed in the air, for he says (translation):

> The cylindrical conidia are typical organs of our fungus whose development is aided or reduced according to the nature of the surrounding medium, not dependent upon it, for they arise constantly as the first product of the germ tubes, in the air as well as in a fluid medium.

While this may be true with *Botrytis bassiana*, which the writer has not yet studied in detail, the blastocysts of Sorosporella obviously can not be homologized with its aerial conidia, because the power which the blastocysts possess of reproducing their kind by yeastlike budding is not shared by the conidia, which furthermore are quite different in form.

De Bary also calls attention to the fact that Audouin (*1*) as early as 1837 probably saw the cutting off of cylindrical conidia in the blood of silkworms infected by *Botrytis bassiana*, and that, according to Robin (*15*), Guérin Ménéville undoubtedly found them, though erroneously claiming that they "arose from the granules contained in the blood corpuscles." On account of their supposed origin Guérin Ménéville called them "Hæmatozoidia," a name which can not be employed at the present time in the light of more recent investigations.

While these earlier authors undoubtedly saw the free-floating fungus cells, Vittadini (*23*), according to De Bary (*2*), first realized their significance because he followed them through the various stages in their development more closely than did any of his predecessors.

Recent literature, however, is entirely lacking in reference to these peculiar free-floating vegetative cells; and only when the older papers were examined by the writer, after these investigations were completed, did it become evident that they were not entirely unknown among entomogenous fungi.

Through the lack of special study of their vegetative phases, as well as ignorance of the older literature, it has been generally supposed in recent years that entomogenous Hyphomycetes as a class vegetate within insects by branching hyphæ. It is possible that such a method of growth obtains in certain entomogenous species, but in *Sorosporella uvella*, *Isaria farinosa*, and *Botrytis bassiana* bodies are formed which seem especially

adapted for reproduction in a liquid menstruum such as insect blood, the circulation of which carries them to all parts of the body cavity and continually supplies them with fresh nutriment.

Thaxter (*21*) in 1888 showed that in certain cases at least the vegetative development of the entomogenous Entomophthorales was carried on not by branching hyphæ, but by "hyphal bodies" which consisted of short, thick fragments of irregular size and form that reproduced by budding, a phenomenon analogous to that described above, though the blastocysts of Sorosporella are regularly elliptical and symmetrical in form.

Because of the similarity of the blastocysts to yeasts and in certain stages even to sporozoans, considerable confusion might result and erroneous conclusions be reached if subsequent stages in the development of the fungus were not known; and in fact so anomalous in character were the blastocysts that considerable care was taken to prove their identity with Sorosporella.

In the course of the cultural experiments described below, it was found that when Uschinsky's solution was inoculated with Sorosporella, a type of yeastlike cell was produced that is quite comparable with that found in the blood of insects. This similarity will at once be evident by comparing Plate 53, B, with Plate 53, A. The former is a photomicrograph of the blastocysts produced on Uschinsky's solution and was made from a water mount, while the latter is from the blood of an infected insect and was made from a slide stained in Erlich's haematoxylin and eosin.

In order to obtain further evidence, however, single blastocysts were removed from the blood of an infected insect by means of Barber's pipette holder and were transferred to nutrient agar.

A small area on the abdomen of an infected insect was washed in alcohol, after which a sterile capillary tube was inserted into the body, and a small amount of blood together with a number of blastocysts was collected. The tube was then removed and its contents ejected upon an inverted sterilized glass slide placed upon a suitable holder on the stage of the microscope. Examination of the drop of blood was then made with the microscope. A place was chosen where a small number of blastocysts occurred, and another sterile capillary tube fastened to Barber's holder was adjusted in such a way that only a few blastocysts were drawn into it. These blastocysts were ejected upon the slide at another place and the process repeated until only one blastocyst was drawn into the tube. This single fungus cell was then ejected upon a hanging drop of culture medium, and a pure growth of Sorosporella resulted.

The operation, though requiring a certain amount of care, was not especially difficult in the present instance, because no extraneous organisms were found in the blood to interfere, and because the blastocysts, which are large, are readily visible and easy to manipulate.

PHAGOCYTOSIS

During the course of these investigations a phenomenon was observed, which, though well known in animal pathology, has received little or no attention from insect pathologists. The discovery of this phenomenon, phagocytosis, in cutworms infected with *Sorosporella uvella* may be of considerable importance from the economic as well as the scientific point of view; and while it is realized that the information given herewith is far from complete, because the subject of phagocytosis and especially its relation to immunity is not clearly understood, it is hoped that the evidence submitted will show clearly that the vegetative bodies of Sorosporella are ingested by the leucocytes of the infected hosts, a condition that has generally been overlooked in previous investigations of insect diseases.

That such a condition has been overlooked is without doubt due to the fact that the vegetative development of entomogenous fungi—that is, those phases which occur within living insects—have never been studied in much detail, although the external reproductive phases are in many cases well known.

When it was discovered that the fungus under consideration vegetates within the blood of infected insects during the early stages of the development, blood smears were made; and after the usual preparatory methods they were stained in Erlich's haematoxylin and eosin. In addition to variable numbers of blastocysts, such slides always show large, spherical, or spindle-shaped, free-floating cells which are of insect origin. Stained preparations showing these cells were submitted to Dr. W. A. Riley, of the University of Minnesota, who kindly informed the writer that he considered them typical blood corpuscles, or leucocytes. He recognized four types, all of which are representative of the Lepidoptera: (1) Proleucocytes, small cells, with large nuclei and little cytoplasm; (2) phagocytes, larger fusiform cells with central nuclei; (3) spherule cells, rounded, vacuolate, and with irregular nuclei; and (4) oenocytoids, large nonphagocytic cells with dense protoplasm.

Of those four types only one, the fusiform cells, is of interest in the present connection, since they alone seem to be phagocytic. These will be considered below under the general terms leucocytes, or blood corpuscles, or the more specific term phagocytes.

When blood smears of cutworms infected by Sorosporella are prepared with the stains noted above, the vegetative budding cells of the fungus are made clearly visible. Not only do they occur often in great abundance, floating free within the blood plasm, but many may also be observed firmly and distinctly imbedded within the cytoplasm of the leucocytes (Pl. 52, B–E), a condition that had escaped attention in water mounts, in which there is of course no differentiation of contents of the leucocytes.

An examination of large numbers of stained slides which were made from several infected insects renders the statement possible that such a condition is of regular occurrence in such insects during the later stages of the incubation period, though it is not possible to state at the present time that every infected cutworm shows this phenomenon.

When treated with the stains noted above, the fungus cells, or blastocysts, are pink. The leucocyte cytoplasm is light blue, whereas the nuclei of the leucocytes are dark blue; hence the fungus cells are clearly differentiated in the blue-stained matrix of the blood cell cytoplasm.

The number of blastocysts contained within a single phagocyte varies from 1 to 15, and so far as can be determined they are in all respects similar to those which occur floating in the blood plasm and may even be seen in the process of budding off new cells (Pl. 52, B, *a*). When gorged with large numbers of fungus cells the phagocytes become abnormal in form and size, though the tail-like protoplasmic appendage can usually be recognized. If but one or two fungus cells are inclosed, however, the form of the leucocyte is unchanged, and its cytoplasm seems likewise to be little affected; for it stains as deeply as in uninfected cells. On the other hand, the cytoplasm of blood corpuscles in which several blastocysts are imbedded seems to be in part destroyed, for it stains feebly. It should be noted, however, that although the nuclei of the infected cells may be compressed or even distorted, they seem to remain intact, staining brilliantly in all tests. The fact that those leucocytes with large numbers of blastocysts imbedded in their substance seem to show evidence of disintegration, together with the fact that in the many preparations examined no evidence of the breaking down of the blastocysts has been observed, renders the conclusion possible that the fungus cells gradually destroy the cytoplasm of the blood cells, which in many cases so completely breaks down that nothing remains.

Such a sequence of events is not unusual in human diseases. There are many cases on record in which it is evident that the phagocytes fail in their attempt to destroy the invading organism, the latter being the more potent; but the act of ingestion of the parasite by the leucocytes is considered nevertheless as phagocytosis, though it may be ineffective so far as the destruction of the parasite is concerned.

In addition to the stained blood smears, paraffin sections were cut of infected larvæ which were killed and fixed in Carnoy's solution. The sections were stained in Erlich's haematoxylin and eosin as before, and in them the phenomenon of phagocytosis was made evident in another way (Pl. 52, J, N).

Complexes such as those referred to are composed of certain cohering cells. Those near the center of the mass are more or less fused, or at least are compressed so closely against one another that their individual identity is hard to determine, though their nuclei remain distinct. Those cells near the periphery of the complex, however, retain their

individuality to some extent; and from an examination of such free or semifree individuals it is possible to determine that they are identical with the free-floating, spindle-shaped cells of the blood, or in other words the phagocytes.

The phagocytes are often arranged in irregularly concentric layers about a mass of free blastocysts, or in other instances infected leucocytes seem to act as a focus around which numbers of blood cells, for the most part uninfected, gather (Pl. 56, A, B). Other sections show what may be called compound complexes, in which there are two or three foci, or centers of attraction, the whole being surrounded by a common envelope composed of several layers of leucocytes. These aggregations, or complexes, occur throughout the body cavity, though more commonly perhaps near the heart or tracheæ; and although they are of various sizes and shapes, they tend to be roughly spherical.

The fusion or coalescence of the amoeboid-like leucocytes recalls at once the similar action of the amoebæ of Myxomycetes in forming plasmodia; and furthermore, as in the Myxomycetes, this coalescence is not accompanied by nuclear fusions, for the nuclei remain distinct.

Since the phagocytic action of the blood corpuscles of susceptible hosts upon the vegetative bodies of Sorosporella had been observed, it was deemed advisable to determine whether or not similar action would occur when the organism was introduced into the blood of non-susceptible hosts.

In inoculation experiments it was found that when the usual methods of infection were employed, silkworms (*Bombyx mori*) and white grubs (*Lachnosterna* spp.) did not succumb to the disease. Sorosporella conidia were therefore injected within specimens of these insects by means of a hypodermic needle. The needle was made from a piece of 4-mm. glass tubing, one end of which was drawn out into a very fine point. An ordinary atomizer bulb was attached to the other end. Conidia in suspension in sterile water were drawn into such sterile tubes, and the body of the host was punctured. Then pressure was applied to the bulb, forcing the conidia into the body cavity. It was found necessary to fasten the insects to some substratum in order to prevent them from wriggling and rupturing the organs around the needle. The needle was then quickly removed; and if the operation was carefully performed, very little blood escaped.

The technic here employed was so crude that it was impossible to to measure very carefully the amount of fluid injected into the larvæ. The diameter of the needles averaged less than 1 mm., and a column of fluid 0.5 cm. long was considered a small dose.

When small injections were made it was nearly always possible to observe conidia in the blood of silkworms and white grubs a few hours after injection, in prepared blood smears; but one or two days after inoculation it was impossible to detect fungus cells of any sort in such

smears. However, although the conidia had disappeared, no evidence of their incorporation by leucocytes was obtained in spite of the fact that these bodies were quite abundant. A few insects so treated were not subsequently punctured to get blood smears but were left unmolested, and after a period of two weeks they were still alive.

It is not possible to say whether the conidia were destroyed by the blood antibodies, or whether they were taken up by fixed cells, which of course would not be seen in blood smears; but in any event their disappearance is suggestive of some such action, and the fact that certain control insects similarly treated survived further indicates that the fungus cells were in some way rendered impotent.

On the other hand, when a large dose of the fluid was injected into silkworms and white grubs, first conidia and then blastocysts could be detected in the blood in due time after injection. The blastocysts appeared within one or two days after injection; and so far as could be determined they reproduced in a way identical with that in susceptible hosts. Similarly the act of phagocytosis was recognized; but, as in susceptible hosts, the phagocytes seemed unable to cope with the fungus, the vegetative bodies of which were formed in enormous numbers. Furthermore the blastocysts rounded up and ultimately formed typical resting-spore aggregations.

In order to determine whether or not there is at first an active phagocytosis in infected susceptible hosts, conidia of Sorosporella were injected into specimens of the semitropical army worm (*Prodenia eridania* Cram.) in small and large doses in a manner similar to that described above.

Blood smears were made from such insects at periods varying from two hours to three days after injection. While it was possible to detect both the fungus cells and the phagocytes in all stained blood smears whether or not such smears were made from insects into which a larger or smaller amount of the fluid was injected, phagocytosis was not observed within two days after inoculation although careful search for infected leucocytes was made. On the other hand, smears made two or three days after injection showed blastocysts incorporated in the phagocytes, although no signs of disintegration of the former could be detected. Furthermore, it is certain that the fungus cells were reproducing rapidly in the blood, for specimens in the 2-celled stage of division, such as are shown in Plate 52, I, were in great abundance.

A detailed discussion of Metchnikoff's discovery of phagocytosis in Daphnia, a crustacean, and his subsequent formulation of the theory of phagocytosis as an explanation of immunity in man and other animals is not within the scope of the present paper, but it seems advisable to consider such phases of this and other theories as may have a bearing on the problem under consideration.

The theory set forth by Metchnikoff (*14*) stated that when an animal is attacked by a hostile organism its blood corpuscles and other proliferating cells of mesodermic origin ingest and destroy the parasite. For a number of years this theory together with the Humoral theory have been considered the two most plausible explanations of immunity; but unfortunately each attempted to explain this phenomenon from a point of view that seemed to be directly opposed to the other, the Humoral theory holding that hostile organisms were destroyed by a body fluid in the nature of a serous exudate.

These differences appear to have been somewhat adjusted in recent years by the work of Erlich, Denys and Leclef, Wright, and others, which has shown the body fluids and the phagocytes to be interdependent in certain human diseases. Wright, notably, proved that the act of phagocytosis is dependent, at least in some diseases, upon the presence of certain substances in the blood, and that these substances (opsonins) act upon the invading organisms, not necessarily killing them, but rendering them susceptible to ingestion by the leucocytes.

It is believed by a certain group of men at the present time that the act of phagocytosis is directly associated with the process of immunization, although the destruction of hostile organisms is brought about not by the phagocytes alone but with the aid of certain body fluids, like opsonins, the nature of which is not fully understood at the present time. Other factors probably are important, such as the potency of the parasite; but these can not be considered here.

While it has been shown in certain human diseases that the leucocytes actually ingest parasitic organisms in a manner comparable to that described above, it is nevertheless well known that such ingestion may end in the destruction of the phagocytes as well as in the death of the organisms, the result apparently depending upon the relative resistance or potency of the leucocyte and parasite.

A study of the blood of infected cutworms has shown that the blastocysts of the parasitic fungus occur within the cytoplasm of the leucocytes. The process by which this position is obtained has not been observed in living material, but from a study of stained individuals it is clearly indicated that amoeba-like pseudopods arise from the phagocytes by means of which the blastocysts are incorporated into the substance of the leucocyte. The occurrence of the phagocytes in aggregations or cysts, however, indicates that some sort of attraction exists between the insect cells and the fungus cells. It is well known that mobile protoplasmic cells exhibit certain definite movements when subjected to mechanical, chemical, or thermal stimuli; and in view of the discovery of Wright, it seems likely that some substance is present in the blood plasm of infected cutworms which not only renders the blastocysts susceptible to ingestion but which also may exert some sort of attraction, perhaps chemotactic, upon the leucocytes, rendering the formation

of aggregations or complexes possible. Furthermore, since the blood corpuscles possess amoeboid movements and are carried from place to place by the circulation of the blood, the formation of the cystlike aggregations is easily understood when this attractive force, perhaps chemotaxis, is considered.

Whatever the force may be, the fact remains that plasmodia are formed, evidently for the purpose of destroying the fungus cells. It has been shown, however, that the latter are more potent than the leucocytes, which ultimately disintegrate; so that while the act of phagocytosis is undoubtedly present and may impede the progress of the disease in cutworms, it can not be considered a successful defensive process in the present instance.

The same statement may be made in regard to those nonsusceptible insects into whose bodies a large number of conidia were injected and which, it will be remembered, were unsuccessful in combating the fungus. When a small number of conidia were injected into the blood of such nonsusceptible hosts, however, it is evident that something was present which was sufficiently potent to render the fungus cells innocuous.

From the discovery of phagocytosis in cutworms parasitized by Sorosporella, it is reasonable to assume that it may be present in other insects attacked by other fungi. It is furthermore not unlikely that the relative potency of the phagocytes and fungi may vary according to the insect and fungus.

Those who have ever attempted to spread entomogenous fungi in the field in an attempt to control insect pests have invariably found many problems relating thereto apparently inexplicable. A further study of phagocytosis may assist in solving these questions. It may explain, for example, why certain individuals or species are immune and others susceptible and why the period of incubation may vary to such a degree in the same disease. From a broader point of view it is not impossible to believe that it will likewise explain why insects appear more susceptible under certain apparently favorable weather conditions than under unfavorable conditions.

CULTURE OF THE ORGANISM

Immediately upon the receipt of the first infected cutworms from College Park, Md., attempts were made to cultivate the organism on artificial nutrients. The plate separation method was at first employed to isolate the fungus; but later it was found, as has been previously noted, that pure cultures could be readily obtained by transferring groups of resting spores directly, with a sterile needle, to sterile plates or tubes.

When first transferred from its natural host to artificial media the organism grows very slowly, and on potato agar it develops less luxuriantly than on any other medium employed. Unfortunately potato

agar was the first nutrient to be used, and it was subsequently discarded as unsuitable. Its use for the first cultures led almost to failure, for some of the plates were discarded after a period of 10 days, when there appeared to be no growth. Fortunately other plates were retained for a longer period; and after 2 weeks a few mycelial strands were observed which could be traced to the spore aggregations, but impurities crept in and crowded out the slow-growing Sorosporella. After numerous fruitless attempts, however, pure cultures were obtained on other more suitable media, from which subcultures have since been made without difficulty.

Fawcett (7), in cultivating the entomogenous Aschersonias of the white fly in Florida, met with the same difficulty; he found that such fungi grew very slowly on artificial nutrients. In fact, he attributes failure in the past in cultivating the species of Aschersonia to the fact that plates were discarded before the organisms had time to develop.

Although a number of media have been employed, several of which are suitable, the writer now uses Molisch's culture media for luminous bacteria[1], modified to some extent, because it is readily made and seems well suited for Sorosporella. This nutrient will hereafter be called Molisch's agar or Molisch's solution.

After the fact was established that the fungus under consideration could be successfully cultivated artificially, a variety of nutrients were employed, on several of which its development was found to differ somewhat from that which occurs in infected insects. Furthermore, single conidia and single resting spores were isolated by Barber's pipette holder and placed in a nutrient drop of agar in a Van Tiegham cell, where development from day to day was easily noted; and pure cultures were subjected to different environmental conditions in order to determine the optimum temperature for growth of the organism as well as its behavior in unusual atmospheres.

To determine the optimum temperature a number of tube cultures were inoculated at approximately the same time. A few were placed in an incubator, a few in a water cooler, and others were kept in the laboratory. The temperature of the incubator varied from 35° to 40° C., that of the water cooler from 18° to 20°, whereas that of the room ranged from 22° to 35° during the month that the tests were in progress. In the incubator the fungus developed feebly though in a normal manner, the thallus covering an area no greater than a square centimeter one month after inoculation. In the water cooler, on the contrary, growth was very luxuriant; and within the same space of time the fungus had spread over the entire surface of the agar slants. The room temperature for the first week or so was quite high, varying between 32° and 34°, and growth of the organism did not keep pace with that in the cultures in the cooler; but later when the room temperature dropped the fungus began to

[1] Water, 1,000 cc.; agar, 15 gr.; sugar, 70 gr.; peptone, 10 gr.; dipotassium phosphate, 0.250 gr.; magnesium sulphate, 0.250 gr.

vegetate more freely, so that at the end of one month cultures left in the laboratory showed nearly as luxuriant a growth as those retained in the water cooler.

The results of these tests indicate a preference of Sorosporella for temperatures varying from 18° to 22° C.; and the fact that such lower temperatures are preferred is not surprising, since the fungus under natural conditions grows in insects which are rarely exposed to heat above 22°, cutworms, for example, being exposed to the air only at night when it is cool, and resting during the day under boards or other débris where the temperature is somewhat lower than in more exposed places.

The fact that the natural vegetative development of the fungus occurs within a liquid menstruum, insect blood, the aeration of which is supposed to be more or less complete through the respiratory action of the tracheæ, led to attempts to cultivate it in artificial liquid media, in certain tubes from which the air was largely removed or replaced by another gas.

In fermentation tubes of Molisch's solution to which the air had free access, however, no gas formation could be detected; but yeastlike vegetative cells were formed abundantly, which were quite similar in size and form to those formed within living insect blood. With the exception of soft Uschinsky's agar and soft Molisch's agar, no other nutrients have been found upon which the fungus vegetates typically by blastocysts; and it seems probable that such a type of development is aided by and perhaps even dependent upon a liquid, or at least a very wet substratum. In addition to the blastocysts which occurred within the nutrient solution and at some distance from its surface, a complex of mycelium filaments, composed of slender, threadlike, septate, sinuous hyphæ, as well as thick filaments with large barrel-shaped cells, was formed at the surface of the solution. The slender, sinuous threads gave rise to conidiophores and conidia; but the thick, barrel-shaped cells first formed spherical, red-colored resting spores, although these bodies later germinated, giving rise to conidiophores and conidia.

To determine whether or not the organism would grow in a partial vacuum, Molisch's solution was placed in ordinary test tubes into which pieces of glass tubing led through perforated rubber corks. The glass tube was then heated about half way from either end and drawn out until it was considerably constricted. The exposed end of the glass tube was then closed with a cotton plug. After sterilization such test tubes were inoculated, and a water pump was attached to the glass tubing with a wash bottle interposed. By allowing the pump to work a few minutes a sufficient amount of air was removed from the test tube to render a fair vacuum. When this was accomplished the constricted portion of the glass tube was rapidly sealed and divided by the application of a Bunsen flame. Several tubes were treated in this manner, in none of which was there at any time afterward any apparent growth of

the organism, but although a limited amount of air seems to be required, free circulation of air is not necessary, because, as noted above, blastocysts were found to develop in fermentation tubes at some distance below the surface of the nutrient solution.

To grow the fungus in an atmosphere of nitrogen instead of air, Buchner's method was employed. Small test tubes filled with Molisch's solution and inoculated were placed in larger test tubes, the latter having been previously filled to a depth of 1 cm. with pyrogallic acid. A few cubic centimeters of a strong solution of caustic soda were then poured quickly between the tubes, the larger of which was immediately sealed with a rubber stopper. The action of the caustic soda upon the pyrogallic acid renders the latter alkaline and in so doing extracts the oxygen from the air, leaving an atmosphere that is composed largely of nitrogen.

When subjected to the conditions outlined above, Sorosporella exhibited no signs of growth whatever; and it may be concluded, therefore, that an atmosphere of nitrogen inhibits growth.

Upon a favorable nutrient and when subjected to the usual cultural conditions in the laboratory, the development of the fungus under consideration takes place somewhat as follows: Mature resting spores when sown either in Van Tiegham cells or in Petri dishes may give rise directly to conidiophores and conidia, as in a drop of water (Pl. 51, L); or at times vegetative, budlike outgrowths are produced, such as are shown at the bottom of M in Plate 51. Such budlike processes produce enormous numbers of cells that remain attached to one another and form a colony. After a short period the members of this colony round up and then give rise to conidiophores and conidia. The conidia, however, when sown on nutrient agar, put forth slender germ tubes (Pl. 51, Q), which after a time become septate and branch profusely. In about 10 days a colony is formed, in which, in addition to such slender hyphæ, there can also be detected large, thick, many-septate fungus filaments, the cells of which are more or less barrel-shaped. In young cultures it can readily be determined that such hyphæ arise as branches of the slender threads. An examination of a colony 2 weeks after the conidia are sown will show, therefore, two types of filaments which are so thoroughly intermingled that it is impossible to ascertain the origin of either. Many of the slender hyphæ increase greatly in length, and those which are at or near the surface of the nutrient agar give rise to sterigmata of the type illustrated in Pl. 52, O. Usually the sterigmata are sessile and occur irregularly on the prostrate hyphæ, which often show a tendency to group themselves in bundles. Occasionally upright, much-branched conidiophores are produced of a type quite similar to those arising from the germinating resting spores.

The thick barrel-shaped cells, however, although at times giving rise to slender, prostrate, vegetative filaments, usually grow more nearly

spherical in form and give rise to other cells of the same nature by a proliferating process, ultimately resulting in a growth of the type illustrated in Plate 51, S. Again, under conditions not fully understood, the elements of such a proliferating colony may become largely disassociated and reproduce by a yeastlike budding process. When either of the last two methods of development is followed the cells normally round up and become thick-walled, forming bodies that have been called resting spores. These bodies are homologous to those found in diseased cutworms and resemble them in the distinctive red color which is visible to the naked eye. On account of the fact, however, that the cells which are differentiated to form resting spores may germinate prematurely in culture and to the fact that the red color is associated only with mature, thick-walled chlamydospores, it should be stated that such pigmentation is not always apparent, or it may appear in limited areas in a culture and not be present elsewhere.

As a result of these diverse methods of development, cultures are produced which to the naked eye present quite different appearances, differences that seem to be due entirely to the tendency of the chlamydospores to germinate in situ at any time during the process of their formation. A comparison of Plate 54, B, with Plate 54, A, will illustrate such different types of growth; and while these photographs are from cultures on beerwort agar and Molisch agar, respectively, dissimilarity is often just as apparent in the same culture. The thallus shown in the latter is convoluted, creamy white, and woolly. It is composed for the most part of immature resting spores which have germinated in situ and have remained attached to one another. In such a proliferating process of growth, if new cells, whether produced laterally or terminally, remain attached to their parents and are incompletely abstricted, a toruloid growth results, the older cells of which germinate in situ before they are matured, forming sterigmata and conidia. When several closely opposed colonies develop in this manner, a thallus is formed which resembles that illustrated in Plate 54, A.

If, on the other hand, the new cells which are formed become largely disassociated from their parents, a growth results that is somewhat different in gross appearance. Under such conditions growth is typically yeastlike, and the elliptical detached cells enlarge considerably by the absorption of nourishment and bud off new cells, so that eventually heaped-up, grapelike bunches are formed. Continued swelling renders the cells nearly spherical in form. Thick walls are deposited. When viewed with the naked eye the characteristic red color invariably associated with fully formed resting spores is at once apparent. Plate 54, B, will illustrate the type of thallus that results from such a method of development, although the photograph was taken a few days too late to show it to the best advantage because germination of the resting spores had already begun in the older portions of the culture.

It should be noted, however, that in some cultures a growth appears such as is shown in Plate 54, B, only to be superseded a few weeks later by one such as is shown in Plate 54, A, which indicates that there is a pronounced tendency on the part of the fungus to change from its natural method of yeastlike development toward a filamentous habit when it is grown on ordinary 2 per cent agar nutrients. When, however, a very soft agar or a liquid nutrient is employed as a substratum, the normal yeastlike habit has a tendency to persist.

Under certain other conditions, however, the tendency to produce free elliptical cells followed by mature resting spores has been very pronounced, as, for instance, in slanted tubes in which the brainlike, convoluted type of development has taken place in a number of instances about the point of inoculation, to be followed some weeks later by the formation of grapelike bunches of mature red resting spores near the bottom of the tube where the agar had shrunk away from the glass.

As noted above, the fungus under consideration was cultivated on a variety of media; and while its development in certain respects was similar on all, differences were observed which are, perhaps, of sufficient worth to be noted.

POTATO AGAR

The development of Sorosporella upon potato agar is always feeble; colonies in flasks do not reach a diameter of 4 mm. by the end of two weeks. After a growth of four or five weeks, a dense, prostrate, dirty white surface mycelial weft is formed, which is composed of very fine, hairlike hyphæ bearing conidia sparsely. The colony at this time may be 1 cm. in diameter. In two or three weeks more it reaches a diameter of 2 to 3 cm. and a dark brownish-colored area appears around its periphery. Growth then seems to cease; the brownish area becomes almost black and extends farther into the medium, forming a dense black ring 2 mm. wide around the colony. A microscopic examination of this area in which no surface mycelium can be seen shows that the discoloration is due to a pigmentation of the subsurface mycelium. True resting spores do not form, though the early stages of the Torula-like budding process can be seen. Transfers of the fungus from such cultures to Molisch's agar have shown that the organism is viable after five months.

MOLISCH'S AGAR

The composition of Molisch's agar, which has been successfully used for growing many species of fungi, is given on page 423. Sorosporella grows more luxuriantly on it than on any other medium employed, except perhaps beerwort agar. A photograph showing its peculiar gross habit after five weeks growth is given in Plate 54, A. When flasks are inoculated by the streak method the fungus may appear in one or both of the two ways that were considered on pages 425–426. Colonies formed by the

yeastlike budding of the fungus cells are at first round, glistening, and white. Later they are red, of a gelatinous consistency, and form grapelike, heaped-up pustules. As noted above, such pustules are composed of mature resting spores, and when they germinate the red color of these bodies is in part obscured by the mass of conidiophores produced. Colonies formed by the toruloid method of growth are convolulted, dense, homogenous, and cream-colored; and after coalescing with other colonies they form a growth such as is shown in Plate 54, A. The crateriform portions of the thallus result largely from a tangential division of the fungus elements within mechanically fixed borders. As noted above, the resting spores in such colonies do not always reach maturity but germinate, giving rise to short, subulate sterigmata, on the tips of which groups of conidia are formed. The sterigmata give the thallus a woolly appearance.

BEERWORT AGAR

The first series of cultures of Sorosporella which were made on beerwort agar developed in a way that has since been observed only occasionally. That is to say, the yeastlike budding process of development, resulting in grapelike aggregations of mature red resting spores, took place in all cultures to the exclusion of all other methods of development. The resting spores did not germinate until they were almost or entirely mature, so that the inoculated flasks presented a striking appearance, the richly developed thallus being exclusively red in color. A portion of a streak culture is reproduced in Plate 54, B; and although the photograph was taken a few days too late, when the resting spores had started to germinate, it is possible to recognize in certain portions of it the gelatinous grapelike clusters of these bodies.

CORN-MEAL AND OAT AGAR

The development of Sorosporella is essentially the same on corn-meal and on oat agar, on both of which it presents some phases that are quite unlike those on other nutrients. At first the writer was inclined to class these nutrients with potato agar as undesirable; but by keeping certain cultures for a long period, a growth resulted that is especially worthy of note. Subsequent inoculations have shown that this peculiar phase of development occurs regularly. As on potato agar, growth is at first extremely slow, practically no surface mycelium being formed around the point of inoculation. After a period of six weeks, however, small wartlike protuberances appeared at the bottom of the tubes, being more abundant in regions where the nutrient had shrunken away from the glass, and at some distance from the point of inoculation with no surface mycelium intervening. The wartlike protuberances, in growing, gradually coalesced, forming a prostrate, crustlike thallus, the red color of which at once indicated the presence of chlamydospores. These resting

spores germinated, not by giving rise directly to sterigmata, but first to hyphæ, those from adjacent spores cohering. As growth proceeded, erect fascicles of hyphæ were thus produced which reached a height of from 1 to 5 mm. Such a synnema is illustrated on Plate 51, G. This fasciation always appears on corn-meal and oat agar if time enough is allowed. It has not been observed on any other nutrient except Uschinsky's. It is worthy of mention because it is identical with the growth that sometimes appears when an infected cutworm is opened up and the inclosed resting spores allowed to germinate on moist sand (see Pl. 55, A). Although such cultures were kept for several months, no further development was observed except that the conidiophores changed from white to a rusty brown in color.

USCHINSKY'S SOLUTION WITH AGAR [1]

For two weeks after inoculation there was no apparent growth of Sorosporella on Uschinsky's solution with agar as a medium. As is often the case with freshly made cultures, however, condensation water was present in the tubes. When the tubes were handled this surface liquid now and again washed against the bit of fungus thallus that was used to inoculate the tubes, and in three weeks' time this surface liquid became milky white in color and upon microscopic examination was found to be full of yeastlike fungus cells. A water mount was made of these cells, a photograph of which may be seen in Plate 53, B. Later the surface liquid changed to a light brown tint and became viscid in consistency. Without changing in other respects it gradually became darker brown and more viscid. The growth was so typically and exclusively yeastlike, liquid, and unlike anything before seen that a bit was transferred to Molisch's agar tubes to determine whether it was an impurity. In due time, however, typical grapelike clusters of red resting spores appeared, indicating that these yeastlike bodies were in fact the organism in question. In the meantime no change had taken place in the original tubes; but at the end of two months the yeastlike elliptical cells began to assume a spherical shape, and when viewed with the naked eye a reddish tinge of the thallus was observed.

The viscid, yeastlike growth gradually disappeared and was superseded by a heaped-up, pustule-like development, more intensely red in color. This growth was composed of spherical, thick-walled resting spores. For a considerable period there was no further change in appearance of the cultures except that as additional resting spores developed the heaping-up process became more conspicuous. Then germination of the resting spores began, and erect fascicles of conidiophores were formed in a manner comparable to that noted above. It is worthy of

[1] Agar agar 15 gr.; asparagin 3 to 4 gr.; ammonium lactate 10 gr.; sodium chlorid 5 gr.; magnesium sulphate 0.2 gr.; calcium chlorid 0.1 gr.; dipotassium phosphate 1 gr. Dissolve in 1,000 cc. water and add 40 cc. glycerin.

remark that throughout the entire vegetative development of the fungus on this medium nothing resembling hyphæ was observed, the entire process having been exclusively yeastlike.

Several months after these tests were conducted a fresh supply of this agar was made up, and on it the fungus under consideration developed in a manner in all respects similar to that described above.

The artificial cultivation of Sorosporella has shown, therefore, that it can be successfully cultivated on a variety of media, and that while its normal method of vegetative development within infected insects is by means of yeastlike budding cells, this habit may be modified by cultivation to such an extent that a semifilamentous growth may be acquired. The cultures also show that when the resting spores reach maturity before germination—a condition that is never accomplished when the toruloid or filamentous habit is followed but which does occur as a result of growth of the disassociated blastocysts on certain nutrients—fascicles of conidiophores arise which recall similar fascicular growths of other insect fungi, such as those of the poorly defined genus Isaria. It should be noted furthermore that no perfect or acigerous stage has been observed in any of the artificial cultures.

INOCULATION EXPERIMENTS

Although the morphological characters, particularly of the resting spores, had been studied to some extent prior to the writer's preliminary work with the fungus in question (*20*), attempts to inoculate insects artificially, either in the laboratory or in the field, were rarely recorded, in spite of the fact that in Russia at least the disease was known to occur under natural conditions out of doors in such abundance and in such a manner as to suggest its infectiousness.

It was deemed advisable, therefore, to perform a series of inoculation experiments to determine, if possible, something of the range of susceptible hosts and the pathogenicity of the organism, as well as to determine a method of infection that could be used in a practical way to inoculate large numbers of insects.

The successful artificial culture of the fungus greatly facilitated the work of conducting these tests, for a constant supply of viable conidia was at all times available. Since it was determined that insects could be readily infected by the fungus from artificial cultures, the latter were invariably used.

It has been shown in the foregoing pages that the vegetative development of the fungus takes place exclusively within the blood of its insect hosts; and it is obvious that to reach the body cavity, the body covering, the tracheæ, or the intestine must be penetrated by the conidial germ tubes. As is well known, insects are covered externally by a layer of chitin, which in certain regions, as for example between the body seg-

ments, is relatively thin. Furthermore, it is generally stated that this layer of chitin extends into the mouth and anus, lining the intestine in both these regions. There is, however, a relatively small portion of the intestine, the mid gut, which is supposed to be of different origin from the fore and hind gut, and which is said not to be lined with this substance. The tracheæ, a ramifying system of spirally reinforced tubes opening externally in the spiracles, are likewise lined with chitin, though this substance is exceedingly thin, especially in the ultimate portion of the tubes.

It is apparent, therefore, that of the regions where the germ tubes might enter the body cavity, all save the mid gut are protected by an inert, resistant substance through which the germ tubes must penetrate. For this reason especial care has been taken to examine the mid gut in prepared sections of inoculated individuals.

It was deemed a matter of considerable interest, both from the scientific and economic points of view, to know exactly how and where infection takes place; and several tests have been carried out with the view of determining this point. There are certain portions of the body wall in which the chitin is comparatively thin, such, for example, as the intersegmental membranes, the flexible leg joints, etc. Furthermore, the glandular openings and hair follicles are apparently less perfectly adapted to resist the penetration of the conidial germ tubes than are most parts of the body. Likewise it would seem quite possible for the small conidia to gain entrance into the body through the spiracular openings of the tracheæ, and once inside to germinate and cause infection. The intestine is, as stated above, lined for the most part with chitin, though this substance is relatively thin in most places and entirely absent in the mid gut.

With these regions of possible infection in mind, artificial inoculations were arranged in such a way that conidia came in contact with all portions of the external body wall and the intestine, and bits of fungus mass were also carefully placed on the tip of a needle directly in contact with the spiracles. In order to introduce the conidia into the alimentary tract, cutworms were fed with clover leaves which had been smeared with the fungus; and to place the conidia in contact with all external parts of the body wall, a bit of conidial agar paste on the tip of a needle was rubbed about over the body, although to facilitate subsequent microscopic examinations of sections of the larvæ it was often applied particularly to the dorsal and ventral medial lines of the body.

To examine the tracheæ after inoculation, it was found advantageous to dissect these organs from the body and mount them directly in alcohol. Although a large number from many insects were examined microscopically, the writer was quite unable ever to find conidia in them. The examination revealed, however, the presence of chitinous spiny bristles at the opening of the spiracles and clustered at various points along the lumina of the tracheæ, and when these were found it became evident that they would effectively prevent the entrance of conidia.

To examine the various parts of the intestine and the body wall, however, it was necessary to cut serial sections of the body. The insects were killed and fixed in Carnoy's solution in periods varying from one to six days after inoculation, after which they were prepared by the usual methods and stained in Erlich's haematoxylin and eosin, such stains having been found of value in differentiating blastocysts within the blood cells.

Although hundreds of sections from a large number of insects were examined, it has been impossible to observe the conidial germ tubes penetrating either the body wall or the intestine. When insects were fed with leaves smeared with conidia, it was possible actually to observe the fungus entering the mouth; yet sections cut of such individuals did not show conidia even in the lumen of the alimentary tract, in spite of the fact that such insects were killed and fixed before the conidia-bearing leaf fragments were voided. In certain slides, however, blastocysts were observed within the folds of a tissue connecting the longitudinal and transverse muscles of the intestine; but it could not be determined whether this position was attained by entrance of the blastocysts from the body cavity or the intestine (Pl. 52, K). Similarly no conidia were observed externally penetrating the body wall of the inoculated insects, though special care was taken to examine the regions noted above and in spite of the fact that in many cases the conidial spore paste was applied in definite regions to facilitate examination.

That the fungus does gain entrance into the body cavity is obvious, however; and as the following tests will show, it seems probable that both the body wall and the intestine may be penetrated by the conidial germ tubes. Inability to observe them is perhaps due to improper time of killing or faulty technic.

To test the parasitism of the fungus and to determine a method of infection that would be adapted to the inoculation of insects artificially on a large scale many tests were conducted of a more general nature than those noted above. A variety of insects, representative of nearly all the larger orders, were inoculated by one or sometimes by all of the methods described below. The hosts used were as follows:

Diptera—larvæ of *Musca domestica* L.

Coleoptera—larvæ of Lachnosterna, larvæ of Elateridae.

Orthoptera—nymphs and adults of grasshoppers.

Isoptera—workers of *Reticulitermes* sp.

Lepidoptera—larvæ of *Feltia jaculifera* Guen., *Feltia subgothica* Haw., *Peridroma saucia* Hübn., *Agrotis ypsilon* Rott., *Noctua c-nigrum* L., *Chloridea obsoleta* Fab., *Hyphantria textor* Harr., *Leucania unipuncta* Haw., *Phlegethontius sexta* Joh., *Bombyx mori* L., and several other members of the family Noctuidae.

Although the fungus has been collected in the field in this country upon two species of Euxoa only, all the Noctuidae used in the laboratory tests, including the corn earworm and army worm, were shown

to be susceptible to the disease. The other species of Lepidoptera—*Hyphantria textor*, *Phlegethontius sexta*, and *Bombyx mori*—did not die of the disease when they were inoculated by the usual methods, yet when conidia were injected into the blood of *Phlegethontius sexta* and *Bombyx mori*, by means of a capillary pipette, death followed; and when such insects were examined, it was found that blastocysts had developed in the blood in a normal manner.

In general, the inoculations were made by one of the three methods outlined below, and after infection the insects were placed in sterile battery jars, partly filled with sterile sand, or if a record of specific insects was desired, they were isolated, one each in small sterile boxes. Control larvæ were kept with all experiments.

DIRECT CONTACT METHOD

Larvæ were placed in flasks or bottles of 500-cc. capacity in which there was a well-developed fruiting surface of the fungus. They were allowed to remain therein for varying periods of time in the several tests and were therefore constantly crawling about over the thallus. It was realized that insects in the field would probably be in contact with the source of infection only for a moment; therefore, in order to simulate in artificial cultures conditions as they occur in the field, the length of time which the insects were allowed to remain in contact with the fungus in the laboratory tests was limited in certain cases to one or two minutes, although in other instances the insects were left in the inoculating flasks for several hours.

In other experiments bits of the fungus thallus were removed from cultures with a sterile needle and rubbed upon the body of the larvæ, or in other instances small portions of the thallus were placed in small boxes on the top of a layer of moist sand and fresh larvæ were then inserted into the boxes. The fungus was allowed to remain in such receptacles for several days so that the cutworms were in daily contact with it.

It is obvious, therefore, that although the methods of inoculation varied in detail, the principle underlying all was the same and consisted in allowing larvæ to come in direct contact with the fungus.

In the discussion of the morphology of the conidia the fact was mentioned that they cohere after abjunction, indicating that a substance is secreted which renders them viscous. This substance is not secreted as profusely as in certain other verticilliacious Hyphomycetes, however; the little heads of conidia were never observed involved in mucus. It is obvious that a substance of this sort would serve also to fasten the conidia to the bodies of insects with which they came in contact; and if certain of the conidia found lodgment in regions suitable for their further development, it is reasonable to believe that infection would result. It is apparent, however, that when the cutworms were allowed

to crawl over the fungus thallus for several hours, the conidia may have been ingested in some numbers; and in such instances the ingested conidia may have produced the disease; but on the other hand, in those instances in which the fungus was applied to the external parts of the body only, it seems necessary to believe that infection was produced by penetration of the body wall by the conidial germ tubes.

The larvæ of all species of cutworms were quite well developed when inoculated, pupating in several instances in a short time. It is worthy of note in this connection that a number of the army worms employed were in the pupal stage at the time of death, and that a few imagos emerged, lived a day or two, and then died of the disease. The occurrence, therefore, of typical resting spores in imagos of the army worm is significant and indicates that the organism, though unable to kill the larvæ and pupæ, passed through the various metamorphic changes of the host and finally caused death after it emerged. The presence of the fungus in the flying adults is furthermore significant, since it suggests the possibility of dissemination of the organism. It should be noted, however, that the wings of the four adults from which the fungus was recovered were imperfectly formed, and other organs were malformed or missing. An antenna of one, for example, was entirely lacking, and a portion of the leg of another was absent, indicating that during the metamorphosis certain of the imaginal tissues were destroyed.

During the summers of 1917 and 1918, 20 tests were performed by the direct contact method, in which many insect species were used; and while it is not desirable to discuss all of them, a few may be considered.

On April 9, 1917, 26 larvæ of *Feltia jaculifera* were inoculated by allowing them to remain in the culture flasks for 12 hours. Thirteen died of Sorosporella on April 20, 8 on April 23, and 2 on April 24. On May 1 when the experiment was closed 3 were alive. There were 12 larvæ in the control dishes, 1 of which died from Sorosporella on April 20. The others were alive on May 1. This larva is the only one that died of the disease in control dishes during the course of all of the experiments.

On August 3, 100 army worms were inoculated by being allowed to crawl over the fungus for two minutes or less. On August 6, 8 larvæ were dead and on August 10, 9 more, from some other cause than Sorosporella. On August 13, however, the fungus was recovered from 9 insects. Metarhizium had killed 2, and 14 had died from unknown causes. On August 15, Sorosporella was found in 20, and 2 were dead from other causes. On August 16, 13 more were dead from Sorosporella; on August 20, 7 emerged, and there were 5 dead from which Sorosporella was not recovered. Finally on the date the test was closed, August 23, 4 adults were found infected with the fungus, as well as 7 pupæ, all of which were filled with resting spores. Of the 25 control larvæ, 5 died from unknown causes and 20 emerged.

In another test, May 9, 15 larvæ of *Feltia* sp. were inserted in as many salve boxes in which were placed moist sand and a bit of fungus

thallus. On May 14, 1 larva died from some unknown cause, but on May 21, 12 larvæ died of the fungus. On the two succeeding days 2 other larvæ died of the same cause. In the control dish in which 10 larvæ were inclosed, 9 were alive on May 23 and 1 had died from an unknown cause.

As an example of those inoculations in which the larval bodies were rubbed with a bit of the fungus, an experiment begun on June 29 with 23 larvæ of *Noctua c-nigrum* may be cited. Thirteen of these were inoculated and 10 were used as controls. On July 6, 3 larvæ died in which the fungus could not be detected. On July 13, 14, and 15, however, 5, 3, and 2 insects, respectively, had died from the disease; and at the close of the test on July 22, 7 larvæ were alive in the control dish, 3 having died before July 15 from unrecognized causes.

In addition to a large number of similar tests that were conducted with noctuid larvæ of various species the direct contact method was also used in inoculating house-fly larvæ, white grubs, wireworms, nymphs and adults of grasshoppers, and white ants, as well as the larvæ of *Hyphantria textor*, *Bombyx mori*, and *Phlegethontius sexta*. The insects in all cases were placed in culture tubes or flasks of the fungus and allowed to remain therein for from 3 to 4 hours, after which they were removed and placed in boxes with suitable food.

The house-fly larvæ, for example, when treated in this manner were found to be covered with conidia when they were removed from the cultures, yet after they had burrowed in the dung of the rearing boxes for a few hours no conidia could be detected with the low power of the microscope. While several specimens, particularly of *Hyphantria textor* and house-fly larvæ, died from unknown causes, the fungus was not recovered from a single inoculated insect of any of the species enumerated above.

The experiments with the susceptible hosts show, however, that under laboratory conditions a high percentage of mortality may be realized and, furthermore, that the death rate is not appreciably higher when larvæ are kept in contact with the fungus for a long time than when they are subjected to infection for a minute or two.

The length of time necessary for the fungus to kill nearly mature cutworms is rarely less than 10 days; and in certain instances it was longer than 10 days, a possible explanation for which is given on page 417. This last summer, however, smaller larvæ in the second and third instars were inoculated for sectioning purposes, and it was found that such insects often succumbed to the disease in 6 or 7 days.

SPRAY METHOD

Ten cc. of sterile water were poured into a culture flask, which was then shaken until a quantity of conidia were in suspension. The liquid was then sprayed upon healthy larvæ by means of a small hand atomizer,

and after inoculation the insects were placed in sterile boxes in the manner described above.

Microscopic examination showed that the liquid held quantities of conidia in suspension. Many conidia therefore must have lodged on the insects, though the liquid did not spread in an even pellicle over the body but gathered in little droplets. Control larvæ were sprayed with sterile water only and were subsequently removed to sterile boxes.

On May 5, 10 larvæ of *Feltia* sp. were inoculated. On May 14, 1 died from an unknown cause; on May 21, 1 died of Sorosporella; and by June 16, 8 were alive. In the control experiment of 10 larvæ, 2 died on May 29 of unknown causes, and the remaining 8 were alive on June 16 at the close of the test.

Seventy-five army worms were inoculated on August 6, and 25 were held as controls. Three of the infected insects died on August 10, but the parasite was not found in them. On August 13, 3 of the inoculated insects, as well as 2 of the control larvæ, died from Metarhizium; 18 of the inoculated specimens had died from unknown causes; and 6 of the control larvæ were dead. On August 17, the parasite was recovered from 5 larvæ. On August 20, 22 of the infected ones emerged; 8 were dead from Sorosporella, and 8 from other causes. Finally on August 23 the remaining 7 of the inoculated army worms were dead from unrecognized causes, and 17 of the control larvæ emerged as adults.

Several other tests of a similar nature were conducted, but it is not advisable to consider them here. The foregoing examples are sufficient to show what may be expected of this type of inoculation. The average mortality of all the tests, however, was somewhat below 30 per cent; and the experiments as a whole serve to corroborate an opinion already formed by the writer in connection with other entomogenous fungi, to the effect that the spray method is of less value in artificially inoculating insects than the direct contact method.

The reason for this is not apparent, because hundreds of conidia must have lodged upon the infected insects. Furthermore, the conidia germinate freely in water and when sprayed upon insects they were apparently in a suitable position to insure infection.

FEEDING METHOD

In order to introduce the fungus into the alimentary tract of cutworms, a conidial agar paste was smeared over clover leaves, which the caterpillars were allowed to eat. But first the leaves were cut into small portions not larger than 5 square millimeters in order to reduce to a minimum the possibility of contact between the leaves and the external parts of the insect's body. Such leaf fragments were then placed in sterilized Petri dishes, into which were also inserted fresh larvæ, one to each dish. Many of the larvæ were hungry, for they had purposely been unfed for several hours; and the process of eating was followed in many

instances until the small bit of leaf was entirely consumed. It is therefore possible to say that the fungus did not come in contact with any external portion of the body except the mouth parts, and the first and second pair of legs, which grasped the leaf, and held it edgewise to facilitate feeding. All conditions under which the experiments were conducted were carried out in such a manner as to preclude the possibility of infection from other sources; and since the fungus at no time came in contact with the body except in the manner stated above, it is obvious that infection was brought about through the ingestion of food.

During the summer of 1918 a number of inoculations were made in this manner. The hosts used were largely cutworms of various species, chiefly *Prodenia eridania* Cram.; and a death rate was obtained in all instances that was as high as in those inoculations which were made by the direct method. A detailed account of one experiment may be given. Twenty-five army worms were fed on August 7 with clover leaves which had previously been smeared with conidia from an agar culture. As a control, 15 larvæ of the same lot were fed with fresh, unsmeared leaves only. On August 10, 3 of the infected specimens and on August 12, 2 of the control larvæ died from unknown causes. On August 16, however, 10 of the infected army worms were dead, in all of which the fungus was recognized; and 3 were dead from other causes. On August 15, 2 and on August 20, 3 larvæ died in the control dishes, but apparently not from the Sorosporella disease. On August 17, 4 and on August 19, 2 of the infected insects died from the disease, and 1 died from which the fungus was not recovered. The experiment was closed on September 3, when 2 larvæ of the infected lot and 8 of the control lot were alive. In spite of the fact, however, that the conidial germ tubes must obviously pass through the intestinal wall, it has been impossible actually to observe them in prepared stained sections.

SUMMARY

(1) The presence of *Sorosporella uvella*, an entomogenous fungus, is recorded for the first time in America.

(2) The previous association of Sorosporella with the Entomophthorales is shown to be erroneous, and the proper position of the organism, among the verticilliacious Hyphomycetes, is designated.

(3) The reproductive bodies are thick-walled resting spores or chlamydospores and thin-walled conidia, the latter being herein definitely associated with the life history of the organism for the first time.

(4) It is shown that yeastlike vegetative cells, existing within the blood of infected insects, are ontogenetically related to other phases in the development of the organism.

(5) There is an ingestion of these vegetative cells by certain of the blood corpuscles (phagocytosis), the process being apparently followed by

the destruction of the phagocytes. This phenomenon has, up to the present time, been overlooked by those investigators who have studied the fungous diseases of insects.

(6) The organism is readily cultivated on artificial nutrients and exhibits two quite different types of growth when grown on favorable media.

(7) In certain cases, both when the fungus was grown on media and when the resting spores were placed in a moist chamber, fruiting structures of the Isaria type developed.

(8) No perfect or acigerous condition has been observed.

(9) The disease caused by the organism is readily transmitted to healthy insects, and in laboratory experiments a mortality of from 60 to 90 per cent may be readily obtained.

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PLATE 51

Sorosporella uvella:

A.—Infected cutworm torn open, exposing the resting-spore aggregations. × 1.2.

B.—A single resting-spore aggregation. × 115.

C.—A portion of a resting-spore aggregation germinating in water, showing promycelial-like germination, and conidia. × 250.

D.—A colony of young resting spores from an infected insect, showing the manner in which they reproduce. × 570.

E.—Isolated mature resting spores, some of which show the ruptured walls of previously cohering spores. × 570.

F.—Mature resting spores germinating in water. × 570.

G.—Isaria-like fascicle of cohering conidiiferous hyphæ which developed when the resting spores of an infected larva were allowed to germinate in a moist chamber. × 300.

H.—Portion of a mature resting-spore aggregation. × 250.

I.—Conidia, or secondary spores. × 570.

J.—Conidia, or secondary spores, germinating. × 570.

K.—Portion of a section through the body of an infected cutworm which had been placed in a moist chamber to induce germination of the resting spores, showing the usual type of conidiophores. × 200.

L.—Mature resting spore germinating in water, showing conidiophore with verticillately arranged sterigmata. × 570.

M.—Mature resting spores germinating on nutrient agar, showing sessile sterigmata and conidia at one place and young resting spores arising by budding at another place. × 570.

N.—Early stages in the germination of mature resting spores in water. × 570.

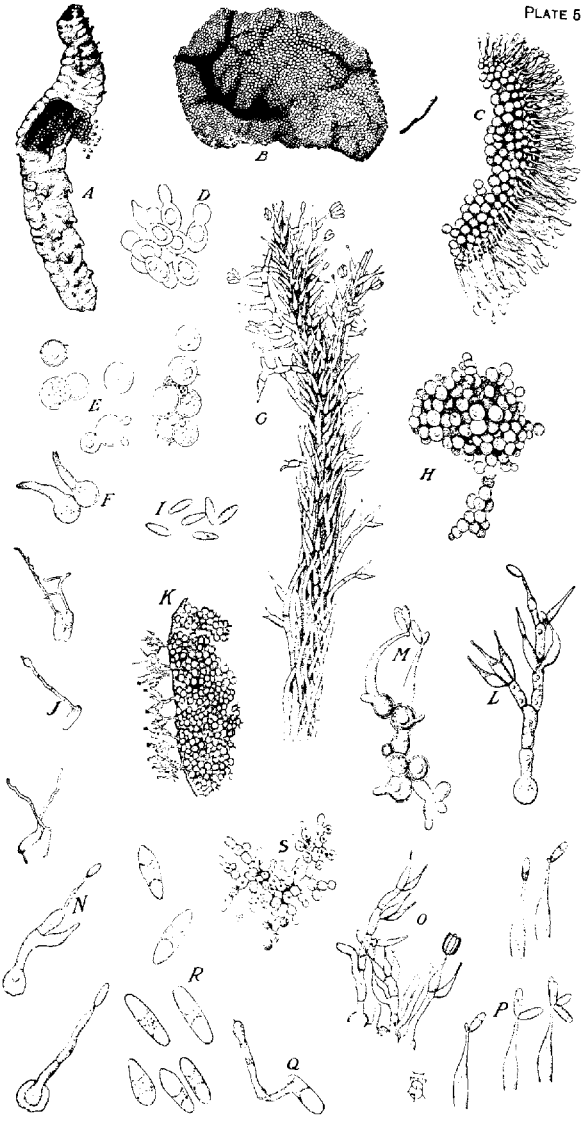
O.—Advanced stages in the germination of resting spores in water. × 570.

P.—Sterigmata, showing method of conidial abjunction. × 570.

Q.—Enlarged view of conidial germination. × 1,050.

R.—Enlarged view of conidia. × 1,050.

S.—Torula-like reproduction in culture. × 570.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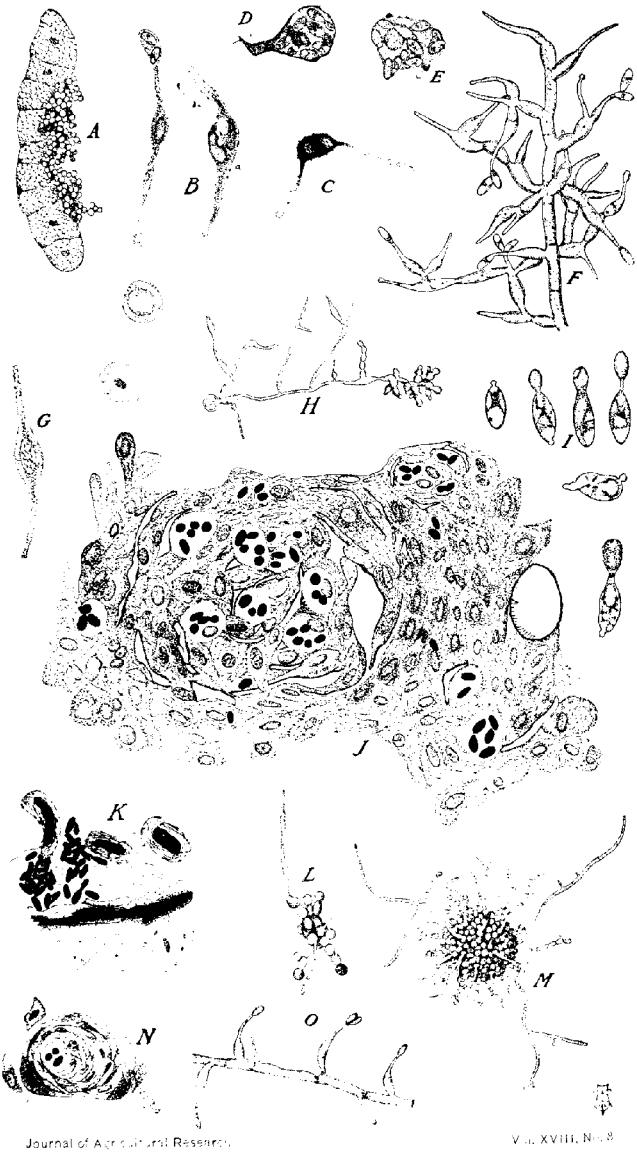
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PLATE 52

Sorosporella uvella:

A.—Portion of a fat body from an infected insect which is being destroyed by an adhering group of young resting spores. × 190.

B, C.—Phagocytes from an infected cutworm with blastocysts of the fungus imbedded in them. × 570.

D, E.—Phagocytes distorted in form and partially disintegrated as a result of the action of the enclosed blastocysts. × 570.

F.—The terminal portion of a complexly branched hypha from nutrient agar, showing sterigmata and conidia. × 570.

G.—Normal blood corpuscles of *Feltia jaculifera*. × 570.

H.—Resting spore germinating on nutrient agar, showing hyphæ, some of which are producing resting spores. × 225.

I.—Blastocysts, showing method of reproduction. × 1,050.

J.—An aggregation of cohering leucocytes in the substance of some of which blastocysts are to be seen. × 550.

K.—Portion of the intestine of an infected *Feltia jaculifera*, showing longitudinal and transverse muscles connected by a frail membrane within the folds of which blastocysts may be seen. × 650.

L.—Colony of young resting spores from a culture, which are giving rise to hyphæ. × 225.

M.—Colony from a Petri dish culture, showing a mass of young resting spores, some of which have germinated in situ by giving rise directly to sterigmata and conidia, and others that are producing hyphæ. × 225.

N.—Small phagocytic complex. × 550.

O.—Portion of one hypha rising from a resting spore that had germinated in a Petri dish culture, showing sterigmata and conidia. × 550.

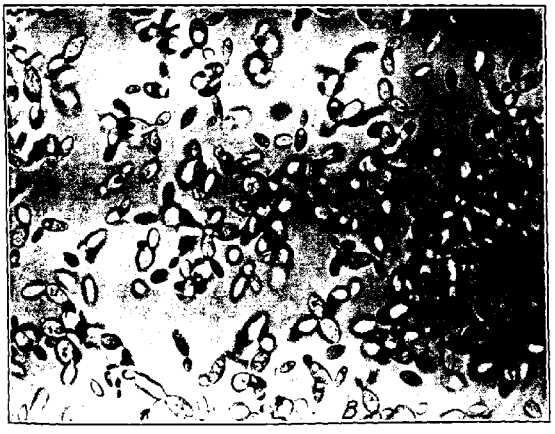
PLATE 53

Sorosporella uvella:

A.—Photomicrograph of a stained blood smear from an infected cutworm, showing a number of free-floating blastocysts and one leucocyte within which are to be seen two blastocysts. × 600.

B.—Photomicrograph of a water mount of blastocysts which developed on Uschinsky's medium after inoculation. × 600.


A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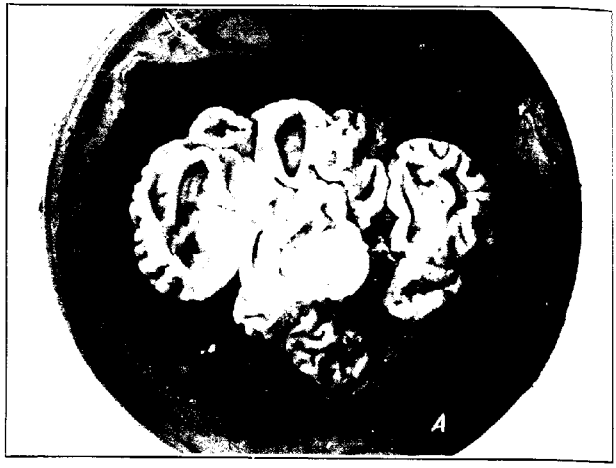

A


B

PLATE 54

Sorosporella uvella:

A.—General appearance of the thallus when grown on Molisch's medium, showing brainlike convolutions and crateriform structure. Natural size.

B.—Streak culture upon beerwort agar, showing shiny gelatinous-appearing, grape-like bunches of mature resting spores, some of which are germinating. × 5.

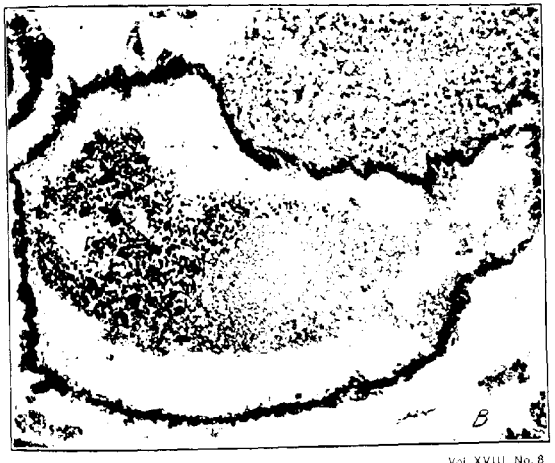
PLATE 55

Sorosporella uvella:

A.—Infected larva of *Feltia* sp. torn open, showing Isaria-like fascicles of conidiiferous hyphæ that sometimes develop when the resting spores are allowed to germinate in a moist chamber. × 10.

B.—Photomicrograph of a stained cross section of the heart, within the cavity of which numerous blastocysts are to be seen. × 125.


A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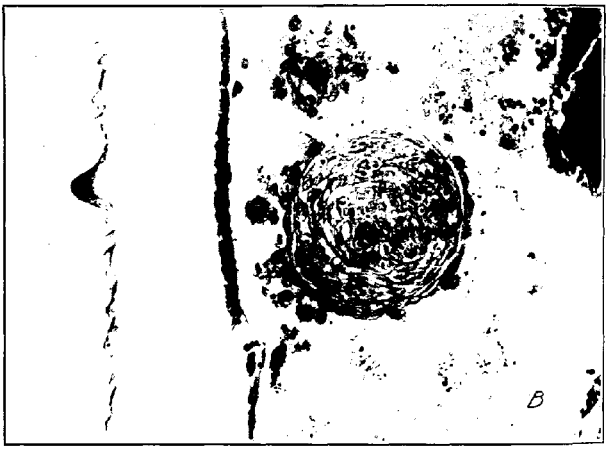
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PLATE 56

Sorosporella uvella:

A, B.—Photomicrographs of phagocytic complexes, showing blastocysts incorporated in the substance of the phagocytes. × 125.

WORK AND PARASITISM OF THE MEDITERRANEAN FRUIT FLY IN HAWAII DURING 1918

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The Mediterranean fruit fly (*Ceratitis capitata* Wiedemann) after its introduction into Hawaii in 1910 soon became the most destructive fruit pest known in the history of the islands. A favorable climate, abundance of host fruits, and the absence of effective control measures allowed it to multiply so rapidly that it soon became established on all the important islands of the Hawaiian group, where it greatly retarded their horticultural development. Immediately after the discovery of the fly in Hawaii, its importance as an enemy was recognized; and the Federal and Territorial governments began to make exhaustive studies of its life history and habits and to experiment with different methods of control.

The use of natural enemies is the only control measure that has been to any great degree successful. Since 1913 the Hawaiian Board of Agriculture has introduced and successfully established in Hawaii four larval parasites of *Ceratitis capitata*—namely, *Opius humilis* Silvestri, *Diachasma tryoni* Cameron, *Diachasma fullawayi* Silvestri, and *Tetrastichus giffardianus* Silvestri. Every year since the establishment of these parasites, the Bureau of Entomology has gathered and published exact data on the degree of control exerted by them over the fruit fly, both as individual species and collectively (*1-4*).[1] This series of publications will be of value to entomologists who are interested in beneficial insects, by giving them definite information regarding the seasonal and yearly success achieved by each one of these four species. The taxpayer, who has paid the expenses connected with the introduction of these parasites, will find in these papers much definite information regarding the benefits he is deriving from his investment. This paper, therefore, is a continuation of these yearly records and gives the extent of parasitism during 1918, the amount of infestation by *C. capitata* for the same period, and, for purposes of comparison, general summaries of parasitism and infestation during 1916 and 1917, taken from literature already cited.

By reference to the data in Table I it will be seen that the infestation during 1918 of a number of host fruits is as great as it was during 1917, and in a few cases considerably greater. All these increases in infestation, with the exception of that in the mango (*Mangifera indica*), have taken

[1] Reference is made by number (italic) to "Literature cited," p. 446.

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place in the most preferred host fruits—that is, in fruits that consistently show high degrees of infestation. The great increase in the infestation of the mango may be accounted for by the fact that some varieties are much more susceptible to fruit-fly attack than others. In 1916 and 1917, infestation records were obtained from 1,317 and 648 fruits respectively, which were collected from a large number of mango trees of different varieties. Many of these varieties showed little or no infestation, and as a result there was a low average infestation for each of these two years. The scarcity of mangoes in 1918 made it impossible to obtain more than 85 fruits, which were of varieties preferred by the fruit fly. This accounts for the high average infestation of 24.4 larvæ per fruit. Those fruits, showing by their comparatively low average infestation that they were not especially preferred as hosts by the fruit fly, contained no more, and in a number of cases contained fewer, larvæ in 1918 than were found in 1917. This lack of increase in infestation of the less-favored host fruits confirms the conclusion drawn from the parasite records of 1917 (*4*)—that, although 50 per cent of the fruit-fly larvæ are destroyed by these parasites and other agencies, little relief is afforded the preferred host fruits, while much benefit is derived from the decreased infestation of the less susceptible host fruits.

This is shown in Tables I to III.

TABLE I.—*Extent of infestation of host fruits by larvæ of Ceratitis capitata in Hawaii during 1918*

Host fruit.	Number of fruits collected.	Number of *C. capitata* larvæ emerging.	Average number of larvæ per fruit.		
			1918.	1917.	1916.
Indian almond (*Terminalia catappa*)	25,558	252,067	9.9	8.0	9.5
Mango (*Mangifera indica*)	85	2,076	24.4	8.1	1.7
Coffee (*Coffea arabica*)	49,130	17,517	.6	.8	.5
Strawberry guava (*Psidium cattleianum*)	24,385	31,692	1.3	2.0	1.6
Black myrobalan (*Terminalia chebula*)	5,664	27,047	4.8	5.9	7.0
Peach (*Amygdalus persica*)	815	18,248	22.4	15.2	20.5
Satin-leaf (*Chrysophyllum olivaeforme*)	1,380	4,376	3.2	3.4	2.0
Rose-apple (*Eugenia jambos*)	1,302	8,568	6.6	8.8	5.5
French cherry (*Eugenia uniflora*)	13,558	13,026	1.0	1.0	.8
West Indian medlar (*Mimusops elengi*)	12,216	30,680	2.5	1.8	5.3
Kamani (*Calophyllum inophyllum*)	888	2,119	2.4	2.4	3.3
Yellow oleander (*Thevetia neriifolia*)	169	1,009	6.0	5.7	3.6
Carambola (*Averrhoa carambola*)	81	74	.9	.6	1.3
Chinese orange (*Citrus* sp.)	7,450	13,349	1.8	1.8	3.1
Guava (*Psidium guajava*)	3,481	29,542	8.5	4.5	6.8
Loquat (*Eriobotrya japonica*)	5,343	9,606	1.8	2.6	.....
Noronhia emarginata	289	323	1.1	.....	.....

Although Table I shows that the infestation of host fruits in general was as great in 1918 as it was in 1917, Table II indicates that parasitism of the larvæ developing in the majority of the abundant host fruits was

higher than in previous years. Notable among these hosts are the Indian almond (*Terminalia catappa*), coffee (*Coffea arabica*), strawberry guava (*Psidium cattleianum*), and French cherry (*Eugenia uniflora*). The most important of the abundant host fruits producing low percentages of parasitism are the mango, kamani (*Calophyllum inophyllum*), Chinese orange (*Citrus* sp.), and guava (*Psidium guajava*). The low parasitism of the larvæ developing in these latter, especially in the guava, large areas of which are growing in all parts of the islands, strongly indicates that these fruits are the source of supply of the large number of fruit flies which cause the continual high infestation of favored hosts.

TABLE II.—*Percentage of larval parasitism of Ceratitis capitata in Hawaii*[a]

Host fruit.	Month of collection in 1918.	Number of larvæ emerging during first 2 to 6 days.	Percentage of parasitism.				
			Opius humilis.	*Diachasma tryoni.*	*Diachasma fullawayi.*	*Tetrastichus giffardianus.*	Total.
Indian almond	February	568	1.8	2.1	.....	0.2	4.1
	March	2,695	37.3	10.7	0.3	4.5	53.0
	April	92	17.2	.....	.....	4.3	21.7
	May	2,134	34.3	36.1	.....	2.9	73.3
	June	8,707	17.7	59.8	.....	1.6	79.1
	July	5,125	2.6	63.2	.1	3.2	69.1
	August	2,746	.8	37.9	.4	5.5	44.6
	September	3,527	1.9	26.6	.4	15.4	44.3
	October	5,748	7.1	25.1	.2	11.0	43.4
	November	6,434	31.4	23.4	.1	13.7	68.6
	December	1,526	22.0	39.6	.3	11.7	73.6
Mango	July	283	2.1	10.6	.4	.7	13.8
Coffee	January	410	2.2	22.9	10.7	.....	35.8
	March	9	22.2	.....	11.1	.....	33.3
	April[b]	1,510	12.0	70.5	8.6	.....	91.1
	September	145	2.1	.....	39.3	.....	41.4
	October	681	3.9	8.3	27.6	.....	39.8
	November	877	16.2	39.1	4.4	.....	59.7
	December	37	5.4	29.7	10.8	.....	45.9
Strawberry guava	January	525	8.8	24.6	2.3	4.4	40.1
	February	286	9.7	19.3	20.4	.5	49.9
	March	593	8.8	6.6	10.8	1.7	27.9
	April	1,531	15.7	13.6	5.1	1.0	35.4
	May	272	33.5	21.0	1.1	4.0	59.6
	July	741	.1	62.1	1.2	2.5	65.9
	August	1,004	.5	45.1	6.9	21.4	73.9
	September	393	2.0	39.0	20.7	6.9	68.6
Black myrobalan	January	1,104	.3	1.2	1.3	.5	3.3
	February	2,348	.6	.5	.3	.1	1.5
	October	190	.....	.....	.....	.....	.....
	November	1,166	.7	2.1	.....	7.9	10.7
Peach	March	733	.....	.....	3.8	.5	4.3
	April	602	.3	2.3	3.7	.2	6.9
	May	581	2.2	5.0	1.0	.7	8.9
	June	307	15.3	9.1	.....	16.3	40.7

[a] The majority of the fruits listed in this table were collected about Honolulu at low elevations. Much of the coffee, however, was collected on the island of Hawaii and in the country districts of the island of Oahu and came from points 1,000 to 2,000 feet above sea level.

[b] The April collections of coffee came from the Kona district of the island of Hawaii.

TABLE II.—*Percentage of larval parasitism of Ceratitis capitata in Hawaii*—Continued

Host fruit.	Month of collection in 1918.	Number of larvæ emerging during first 2 to 6 days.	Percentage of parasitism.				
			Opius humilis.	*Diachasma tryoni.*	*Diachasma fullaways.*	*Tetrastichus giffardianus.*	Total.
Rose-apple	May	186	4.3	9.1	.....	.....	13.4
	July	362	.8	54.4	.....	2.2	57.4
	August	836	.6	28.0	.....	1.0	29.6
	September	386	8.0	41.5	.....	5.7	55.2
Satin-leaf	January	534	20.4	3.7	5.8	.2	30.1
	February	123	18.7	7.3	8.1	3.2	37.3
French cherry	January	532	4.9	18.8	7.1	.2	31.0
	February	83	4.8	1.2	.....	.....	6.0
	March	42	2.4	.....	.....	2.4	4.8
	June	1,212	1.1	71.8	5.9	.6	79.4
	July	813	3.1	49.9	18.7	.9	72.6
	August	265	.4	46.4	38.8	4.5	90.1
	November	175	8.6	3.4	.....	.....	12.0
	December	86	20.9	7.0	.....	1.2	29.1
West Indian medlar	March	77	.....	.....	.....	.....	.....
	April	139	.....	.....	.....	.....	.....
	May	1,220	2.8	.....	.....	.4	3.2
	June	1,360	2.8	9.1	.1	.7	12.7
	July	212	.....	7.1	.....	1.4	8.5
Kamani	January	466	.....	.8	.....	.2	1.0
	February	42	.....	.....	.....	.....	.....
	March	120	.8	1.7	.8	.....	3.3
	April	14	7.1	.....	.....	.....	7.1
	July	85	.....	38.8	.....	.....	38.8
Yellow oleander	September	23	.....	13.1	.....	.....	13.1
	October	266	5.6	4.5	8.6	3.8	22.5
Carambola	January	15	.....	13.3	.....	13.3	26.6
	February	18	5.6	.....	.....	5.6	11.2
	November	7	.....	.....	.....	.....	.....
Chinese orange	January	577	2.2	1.7	.2	.5	4.6
	February	95	.....	6.3	.....	.....	6.3
	March	135	.7	11.9	.....	.....	12.6
	April	71	.....	.....	.....	.....	.....
	May	41	2.4	.....	.....	.....	2.4
Guava	February	136	9.6	9.6	.....	.7	19.9
	April	716	5.8	1.5	.4	.1	7.8
	May	1,422	6.0	9.8	.2	2.7	18.7
	June	2,802	2.2	27.4	1.3	11.8	42.7
	July	1,158	.8	18.9	1.2	9.8	30.7
Loquat	January	1,056	4.4	12.3	17.4	.8	34.9
Noronhia emarginata	July	44	.....	11.4	.....	.....	11.4

TABLE III.—*Total parasitism of all larvæ of Ceratitis capitata collected in Hawaii during 1918, arranged by months*

Month.	Number of larvæ.	Percentage of parasitism.						
		Opius humilis.	*Diachasma tryoni.*	*Diachasma fullawayi.*	*Tetrastichus giffardianus.*	Total for 1918.	Total for 1917.	Total for 1916.
January	5,219	4.8	9.6	6.2	0.8	21.4	59.0	6.98
February	3,600	2.3	2.5	1.6	.2	6.6	32.9	19.5
March	4,404	24.1	7.9	2.3	3.2	37.5	63.5	14.7
April	4,675	10.3	27.8	5.0	.4	43.5	43.3	37.64
May	5,854	16.5	17.3	.2	2.0	36.0	40.9	26.69
June	14,388	11.8	48.6	.8	3.7	64.9	36.1	27.81
July	8,827	2.0	52.2	2.1	3.6	59.9	51.0	18.52
August	4,850	.7	38.1	3.8	7.9	50.5	33.1	37.5
September	4,471	2.4	28.0	3.4	13.3	47.1	52.4	45.2
October	6,885	6.5	22.0	3.2	9.4	41.1	45.2	44.3
November	8,659	25.3	21.7	.5	11.2	58.7	72.3	44.3
December	1,648	21.5	37.7	.4	10.9	70.5	34.2	44.1
Average for 1918	63,480	12.4	34.6	2.6	6.2	55.8	.....	.....
Average for 1917	72,139	12.7	20.3	7.3	7.2	.....	47.5	.....
Average for 1916	83,304	17.2	13.3	2.1	.6	.....	.....	33.2

Previous publications (*1–4*) give data that show the consistent ascendancy of the parasite *Diachasma tryoni* over *Opius humilis* during the warmer months of the year and the predominance of *O. humilis* in the cooler months. This interchange in the effectiveness of these two parasites is due to the ability of *D. tryoni* to destroy *O. humilis* when both occur in the same host larva, coupled with the decreased activity of the former during the cooler months (*5*). Records of parasitism for 1916 and 1917 (*3–4*) show that *O. humilis* gained this predominance for five and three months, respectively; while during 1918, as shown by Table III, this species was able to gain the ascendancy for only two months—March and November. This yearly decrease in the effectiveness of *O. humilis* is directly due to the yearly increase in numbers of *D. tryoni*, which is shown by the average yearly parasitism records given at the bottom of Table III. The average parasitism by *D. tryoni* increased from 13.3 larvæ to each fruit in 1916 to 20.3 in 1917 and to 34.6 in 1918, while the average parasitism by *O. humilis* consistently decreased. Although the parasitism by both *D. fullawayi* and *Tetrastichus giffardianus* was less in 1918 than in 1917, the total percentage of parasitism for the last year, on account of the increased effectiveness of *D. tryoni*, had increased 8.3, making the total parasitism for 1918, 55.8 per cent of all the fruit-fly larvæ under observation.

The carefully recorded data concerning the activities of the Mediterranean fruit-fly parasites in Hawaii show that their value as destroyers of this pest has consistently increased each year since their introduction,

until in 1918 they caused the destruction of considerably more than half of all the fruit flies developing in fruits about Honolulu. This great decrease in the numbers of this pest has been of direct benefit to the people of Hawaii by greatly decreasing the infestation of the fruits less susceptible to fruit-fly attack, since this class contains the majority of fruits of commercial value. It has been of value also to the fruit growers of the mainland United States by greatly decreasing the danger of the introduction of the fruit fly there.

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CYANOGENESIS IN SUDAN GRASS: A MODIFICATION OF THE FRANCIS-CONNELL METHOD OF DETERMINING HYDROCYANIC ACID

By Paul Menaul, *Assistant Chemist*, and C. T. Dowell, *Chemist, Oklahoma Agricultural Experiment Station*

There have been a few cases reported from this and other States of the poisoning of cattle while pasturing on Sudan grass. Prof. H. D. Hughes of the Iowa State College was so kind as to send us information which he had obtained on the subject. It seems that no determinations of the hydrocyanic acid in Sudan grass have been made except those made by Francis[1], and for that reason the writers decided to determine the percentage of the acid present in the grass at several stages of its growth.

Our first sample was cut on June 16 when the grass was 15 inches high. Other samples were taken at intervals of one week thereafter until the grass was cut for hay. There had been plenty of rain, and the grass had grown very rapidly.

The method used for determining the acid was that employed by one of the writers[2] in determining the amount of the acid in kafir. The grass was cut fine, bruised thoroughly in an iron mortar, and then covered with water in a flask and kept at 40° C. for two hours. After this it was made more strongly acid with tartaric acid and distilled into 30 cc. of a 2 per cent solution of sodium hydroxid. The cyanid was precipitated as Prussian blue. After being burned in a muffle furnace the iron oxid was weighed, and from this weight the weight of hydrocyanic acid was calculated. One or two of the last determinations were made by the use of the Francis-Connell method[3] as modified by the writer. The results are shown in Table I.

From this table there is seen to be a decrease in the hydrocyanic acid with growth. This would be expected since similar results have been obtained with other varieties of sorghums. More of the acid is present in the leaves than in the remainder of the plant. This has been found by others to be true for other plants. A number of determinations which are not given in the table show that there is more hydrocyanic acid in the plant in the morning than in the afternoon. The writers

[1] Francis, C. K. Poisoning of live stock while feeding on plants of the sorghum group. Okla. Agr. Exp. Sta. Circ. Inform. 38, 4 p. 1915.

[2] Dowell, C. T. Cyanogenesis in Andropogon sorghum. *In* Jour. Agr. Research, v. 16, no. 7, p. 175–180. 1919.

[3] Francis, C. K., and Connell, W. B. The colorimetric method for determining hydrocyanic acid in plants with special reference to kafir corn. *In* Jour. Amer. Chem. Soc., v. 35, no. 10, p. 1624–1628. 1913.

are not aware that this observation has been made before. A comparison of the results obtained here with those given in the literature for kafir and other varieties of grain sorghums shows that Sudan grass contains about one-third as much hydrocyanic acid as do the grain sorghums.

TABLE I.—*Percentage of hydrocyanic acid in Sudan grass at different stages of growth*

Time of cutting.	Percentage of dry matter.	Percentage of hydrocyanic acid in leaves on dry basis.	Percentage of hydrocyanic acid in whole plant on dry basis.	Percentage of hydrocyanic acid in leaves on fresh basis.	Percentage of hydrocyanic acid in whole plant on fresh basis.	Remarks.
June 16	18.2	.....	0.0579	.....	0.0105	Height of plant, 15 inches. Plant was mostly leaves.
23	19.5	0.0465	.0274	0.0090	.0053	
30	23.7	.656	.0291	.0155	.0069	Rain of 0.53 inches on June 28.
July 7	31.0	.....	.0094	.....	.....	Grass cut between July 7 and 14.
14	31.0	.....	.....	.....	.0052	Grass cut in the morning.
					.0035	Grass cut in the afternoon.
31	22.0	.....	.....	.....	.0059	Second growth, 18 inches high.

MODIFICATION OF THE FRANCIS-CONNELL METHOD OF DETERMINING HYDROCYANIC ACID

Viehover and Johns[1] have criticized the Francis-Connell colorimetric method of determining hydrocyanic acid, pointing out (1) that the equilibrium of the reaction upon which the method is based is very sensitive to the presence of electrolytes and hence that the intensity of the color due to the ferric sulphocyanid is varied greatly by salts and (2) that a part of the sulphocyanic acid is volatilized in boiling the acid solution to remove the colloidal sulphur. Johnson[2] criticized the Viehover-Johns colorimetric method and used the Francis-Connell method after modifying it. The Viehover-Johns method is objectionable on account of the fact that the intensity of the color due to the Prussian blue varies with temperature and is changed by electrolytes. The Francis-Connell method would seem to be the better method if the objections pointed out by Viehover and Johns could be overcome. Johnson's modification of the Francis-Connell method makes the process too long, and the solutions used are more sensitive to electrolytes than are those used in the original method. The writers sought to modify the method and eliminate the objectionable features.

[1] VIEHOVER, Arno, and JOHNS, Carl O. ON THE DETERMINATION OF SMALL QUANTITIES OF HYDROCYANIC ACID. *In* Jour. Amer. Chem. Soc., v. 37, no. 3, p. 601–607. 1915.

[2] JOHNSON, Maxwell O. ON THE DETERMINATION OF SMALL QUANTITIES OF HYDROCYANIC ACID. *In* Jour. Amer. Chem. Soc., v. 38, no. 6, p. 1230–1235. 1916.

PREPARATION OF THE STANDARD SOLUTION

Ten cc. of a solution containing 5 mg. of hydrocyanic acid as potassium cyanid were placed in an evaporating dish, and 1 cc. of concentrated yellow ammonium sulphid and one drop of concentrated sodium hydroxid were added. This was slowly evaporated to dryness on a water bath by passing a current of air over the dish by means of an electric fan running at low speed. The residue was heated to 130° C. for five minutes then dissolved in 10 cc. of warm water acidified with dilute hydrochloric acid, two or three drops being added in excess. A 15 per cent solution of cadimum chlorid was added drop by drop until the sulphid ceased to form, and then a 10 per cent solution of ferric chlorid was added until the red color was permanent. This solution was then filtered through a moistened paper and 5 cc. of 10 per cent solution of ferric chlorid added to the filtrate. The volume was then made up to 100 cc.

PREPARATION OF THE UNKNOWN SOLUTION

One cc. of concentrated yellow ammonium sulphid was added to the distillate obtained by the process described above and then evaporated slowly on the water bath as in the preparation of the standard solution. The temperature was kept at about 70° C. The residue was treated as in the preparation of the standard solution. The standard and unknown solutions were then compared by means of a Bock-Benedict [1] colorimeter.

The method was tested by comparing a solution of potassium cyanid, which had been standardized by precipitation with silver nitrate, with a solution of potassium sulphocyanid, which had been standardized by the Volhard method. Two or three drops of dilute hydrochloric acid were added to each solution before the addition of the ferric chlorid.

The purpose of heating the residue to 130° C. was to prevent the colloidal sulphur from going back into solution. The maximum intensity of color was obtained with the concentration of ferric chlorid used by the writers. Johnson [2] used 2 cc. of a 1 per cent solution of ferric chlorid to the hundred and Francis-Connell [3] used 1 cc. of a 10 per cent solution. The writers found, as did Johnson, that the addition of hydrochloric acid to a solution containing but 2 cc. of a 1 per cent solution of ferric chlorid to the hundred increased the intensity of the color, and that the addition of potassium chlorid to such a solution had a bleaching effect. No change in the intensity of the color could be detected by either of the writers after the addition of the acid and salt to their solution. The results obtained by Johnson are what would be expected when dilute solutions are used.

[1] BOCK, Joseph C., and BENEDICT, Stanley R. A NEW FORM OF COLORIMETER. *In* Jour. Biol. Chem. v. 35, no. 2, p. 227–230, 3 pl. 1918.

[2] JOHNSON, Maxwell O., 1916. OP. CIT.

[3] FRANCIS, C. K., and CONNELL, W. B., 1913. OP. CIT.

SUMMARY

(1) Sudan grass was found to contain about one-third as much hydrocyanic acid as is found in the grain sorghums. The quantity is greatest in the young plant and decreases rapidly as the plant matures.

(2) It was found that the colloidal sulphur formed by the Francis-Connell method could be removed by evaporating the solution to dryness and heating the residue to 130° C. for five minutes.

(3) When 5 cc. of a 10 per cent solution of ferric chlorid were used in 100 cc. of solution no observable change in intensity of color was produced by the addition of small amounts of hydrochloric acid and potassium chlorid either together or separately.

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